

Transcriptional repression of human *cad* gene by hypoxia inducible factor-1 α

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ABSTRACT

***De novo* biosynthesis of pyrimidine nucleotides provides essential precursors for DNA synthesis and cell proliferation. The first three steps of *de novo* pyrimidine biosynthesis are catalyzed by a multifunctional enzyme known as CAD (carbamoyl phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase). In this work, a decrease in CAD expression is detected in numerous cell lines and primary culture human stromal cells incubated under hypoxia or desferrioxamine (DFO)-induced HIF-1 α accumulation. A putative hypoxia response element (HRE) binding matrix is identified by analyzing human *cad*-gene promoter using a bioinformatic approach. Promoter activity assays, using constructs harboring the *cad* promoter (–710/+122) and the –67/HRE fragment (25-bases), respectively, demonstrate the suppression of reporter-gene expression under hypoxia. Suppression of *cad*-promoter activity is substantiated by forced expression of wild-type HIF-1 α but abolished by overexpression of dominant-negative HIF-1 α . A chromatin immunoprecipitation assay provides further evidence that HIF-1 α binds to the *cad* promoter *in vivo*. These data demonstrate that the *cad*-gene expression is repressed by HIF-1 α , which represents a functional link between hypoxia and cell-cycle arrest.**

INTRODUCTION

Hypoxia is an environmental stress that trigger with important implications in the pathogenesis of major causes of mortality including cancer, cerebral and myocardial ischemia, pre-eclampsia, and chronic heart and lung diseases. In recent years, many studies have shown that hypoxia can inhibit or

even completely abolish cell proliferation (1–8), which is directly involved in the chemoresistance and radioresistance of hypoxic tumors because, chemo- and radiotherapy are efficient only against rapidly dividing cells. Thus, understanding the mechanisms responsible for cell-cycle arrest under hypoxic stress might help to circumvent these two forms of resistance.

The expression of hypoxia-responsive genes is regulated primarily by the transcription factor hypoxia-inducible factor (HIF). HIF consists of an inducible α subunit and a constitutively expressed β subunit. Three variants of HIF- α (HIF-1 α , -2 α and -3 α) have been identified (9); all of them dimerize with HIF-1 β (also known as aryl hydrocarbon nuclear translocator, ARNT) to form a heterodimeric functional unit. HIF-1 α is expressed in most, if not all, human tissues (10), while HIF-2 α and HIF-3 α are expressed in more restricted tissues and developing stages such as the fetal lung and vascular endothelium (11–13). In addition, HIF-1 α appears to play a general role in the transcriptional regulation of all cells in response to hypoxia, whereas HIF-2 α and HIF-3 α play more limited or specialized roles in oxygen homeostasis.

Recent studies (1–3) have demonstrated that hypoxia may lead to apoptosis of some transformed cell lines, while non-transformed cells are viable under hypoxic condition but are arrested in the G1/S-phase. Upregulation of HIF-1 α caused by hypoxia inhibits cell-cycle progression in most normal and cancer cells (4–8). However, the mechanism of HIF-1 α -induced cell-cycle arrest has not been uniformly resolved. Hypoxia-induced cell-cycle arrest causes the elevation of checkpoint proteins such as p53, p21 and p27 in direct relation to the expression level of HIF-1 α (3,14). In contrast, a recent study demonstrated that embryonic fibroblast cells without p21 and p27 are capable of hypoxia-induced S-phase arrest, but that re-entry into the cell-cycle after reoxygenation is delayed (8). Most importantly, direct evidence of the transcriptional regulation of these checkpoint proteins by HIF-1 α has never been demonstrated. Therefore, it is hypothesized that the elevation of checkpoint proteins under hypoxia is the

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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secondary barrier of cell-cycle arrest and that a nucleotide shortage is the primary cause of the arrest (1).

The *de novo* biosynthesis of pyrimidine nucleotides provides essential precursors for DNA synthesis and cell proliferation. Pyrimidine biosynthesis is not only a prerequisite for cells to enter the S-phase, but also it plays a dominant role in regulating cell-cycle progression due to the increased demand for nucleotides for DNA synthesis in rapidly proliferating cells. The first three steps of *de novo* pyrimidine biosynthesis are catalyzed by a multifunctional cytoplasmic enzyme known as CAD (carbamoyl phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase) (15,16).

Over the past decade, extensive advances have been made in elucidating the mechanisms of the *de novo* biosynthesis of pyrimidine nucleotide in mammalian cells. The cloning of the human *cad* gene and the characterization of its properties provided the major breakthrough for our understanding of the principles and regulation of pyrimidine biosynthesis in cancerous and non-cancerous cells. It is clear that CAD catalyzes the rate-limiting step in the *de novo* pyrimidine synthetic pathway (17), and that it therefore controls the rate of DNA synthesis. Transcriptional upregulation of *cad* promoter by growth factors and steroids that ultimately leads to an increase in cell proliferation has been reported. Mitogen-activated protein kinase (MAPK) and the transcription factor c-Myc mediates the growth-factor-induced CAD overexpression, and estrogen upregulates *cad*-promoter activity via the binding of ER and SP1 to the GC box of *cad* promoter (18,19).

The biosynthesis of pyrimidine nucleotides is inhibited by hypoxia, which ultimately leads to reduced DNA synthesis, hence inhibiting cell-cycle progression (7,20,21). Addition of exogenous pyrimidine nucleotides causes re-entry into the S-phase and initiates replication in hypoxic cells (7). Because CAD controls the first three enzymatic activities of pyrimidine nucleotide biosynthesis, reducing pyrimidine nucleotide production in hypoxic cells may be mediated by inhibiting CAD expression. Furthermore, HIF-1 α is the master transcription factor in regulating hypoxia-responsive gene expression; thus, we hypothesize that HIF-1 α may directly inhibit *cad* under hypoxia condition. This study aims to investigate regulation of CAD by HIF-1 α using both *in vitro* and *in vivo* approaches.

MATERIALS AND METHODS

Cell culture and hypoxia treatment

Cell lines were maintained in specific culture media recommended by the American Type Culture Collection (ATCC), and were supplemented with 10% fetal bovine serum (FBS). The human endometrial stromal cells were purified and cultured in DMEM/F12 medium with 10% FBS, as previously described (22,23). Between 18–24 h before undergoing hypoxia, cells were plated at a density of 5×10^5 cells per 30 mm glass dish. The medium was changed ~ 1 h before treatment to assure an adequate amount of nutrient and growth factor. Hypoxic treatment was carried out in an incubator with 1% O₂, 5% CO₂ and 94% N₂, or with desferrioxamine (DFO), as indicated. Oxygen concentration was monitored using an oxygen electrode (OS1000; Oxygen Sensors, Inc, Frazer, PA).

RNA isolation and real-time RT-PCR

Total RNA was isolated from cells using a kit (RNeasy Mini Kit; Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentrations were determined using UV-absorption at 260 nm, and then subjected to reverse-transcription at 42°C for 60 min, denaturing, and PCR amplification using a thermal cycler (ABI 7900; Applied Biosystems, Foster City, CA). To determine the amounts of CAD transcript, real-time RT-PCR was conducted. In this reaction, SYBR Green I was added to the PCR master mix and served as a fluorescence source for laser detection. The cycling conditions were 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. The reaction data were expressed as copies/ μ g of RNA, after converting the number of cycle thresholds (*Ct*)-the PCR cycle number at which the fluorescent signal in each reaction reaches a preset threshold above background-to absolute value according to the standard curve. A dissociation curve was created using the built-in melting-curve program to confirm the presence of a single PCR product. 18S rRNA was used as an internal control, for which the primer sequences were previously reported (24).

Plasmids, transfection, and promoter-activity assays

Human HIF-1 α CEP4/HIF-1 α (ATCC Cat no.: MBA-2) and dominant-negative form HIF- α pCEP4/HIF-1 α DN (ATCC Cat no.: MBA-7) plasmids were purchased from the American Type Culture Collection (ATCC, The Johns Hopkins University, Baltimore, MD). Human *cad* promoter (–710/+122) was cloned to pGL3 basic vector (Promega Corp., Madison, WI) using PCR with a pair of primers: (forward: 5'-ctagctagctagAAAGGAGAGCCACAAGACCA-3'; reverse: 5'-ccgctcgagcggGGAAGGACTGCAAACTCCAC-3'), which contained NheI and XhoI restriction sites (underlined), respectively. Alternatively, two 25-base oligonucleotides: (forward: 5'-ctag-CCGCCCCCTTACGTGCCCCGGCCCCGC-3', reverse: 5'-tcga-GCGGGGCCGGGCACGTAAGGGGCGG-3') corresponding to human *cad* HRE matrix (–76/–52) were synthesized and cloned into SV40-driven pGL3 plasmid after annealing. Mutation of the putative HRE sequence was achieved by replacing the bases ACGTG with ATTAG.

Cells were placed on 24-well plates for luciferase assays. Commercial plasmids containing a CMV-driven β -galactosidase reporter system (Promega, Madison, WI) were transfected into the cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). After transfection, the cells were allowed to rise and incubated in serum-free DMEM/F12 medium for 6 h. The medium was then changed, and cells were subjected to hypoxic treatment for the indicated time. Luciferase assays were done using a kit (Dual Luciferase Reporter Assay System, Promega) according to the manufacturer's instructions. Briefly, 100 μ l of luciferase substrates were added to 20 μ l of lysate, and luciferase activity was measured using a 20/20 luminometer (Turner Designs, Sunnyvale, CA). Each luciferase assay experiment was performed in duplicate and repeated as indicated in the figure legends.

siRNA

Short interference RNA (siRNA) against human HIF-1 α was synthesized by Qiagen. Sequences for siHIF-1 α

are: 5'-r(CUGAUGACCAGCAACUUGA)d(TT)-3' and 5'-r(UCAAGUUGCUGGUCAUCAUCAG)d(TT)-3'. For negative control, two ribonucleotides with scrambled sequences 5'-r(AGUUCAACGACCAGUAGUC)d(TT)-3' and 5'-r(GACUACUGGUCGUUGAACU)d(TT)-3' were also synthesized. The ribonucleotides were dissolved in the siRNA suspension buffer (Qiagen), heated to 90°C for 1 min, and incubated at 37°C for 60 min. The annealed siRNA (5 µg) was used to transfect cells according to manufacturer's instruction (Qiagen). After transfection, cells were incubated with or without 10 mM DFO for 8 h and subjected to RNA isolation and real-time RT-PCR quantification as described above.

Chromatin immunoprecipitation (ChIP)-PCR assay

The protocol used was as described before (24) with modifications. In brief, proteins (HIF-1 α) and DNA in normoxia, hypoxia, or DFO-treated cells (1×10^6 cells) were cross-linked by incubation for 10 min at 37°C with a final concentration of 1% formaldehyde. After aspiration of the formaldehyde, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 µg/ml each of aprotinin, and pepstatin A) and scraped into a conical tube. The lysate was then centrifuged in a desktop centrifuge (Model 5415R, Eppendorf, Hamburg, Germany) for 5 min at 500 g at 4°C, resuspended in 400 µl of lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1)], and placed on ice for 10 min. Genomic DNA was sheared to lengths of 0.5–1 kb by sonicating the cell lysate, then debris was removed by centrifugation, and the supernatant diluted with 1 ml of ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 16.7 mM NaCl and proteinase inhibitors (pH 8.0)]. Two percent of the diluted lysate was kept for input control. The chromatin solution was pre-cleared with mixtures containing BSA, salmon sperm DNA and protein A Sepharose. Anti-HIF-1 α antibody or rabbit IgG (for negative control) was added to the supernatant fraction and the mixture was incubated overnight at 4°C with rotation. Then, 60 µl of salmon sperm DNA/Protein A agarose slurry was added to the mixture and incubated for 1 h at 4°C with rotation. The protein A agarose/antibody/HIF-1 α complex was pelleted by gentle centrifugation (400 g at 4°C for 1 min) in a desktop centrifuge (Eppendorf, Model 5415R). The pellet was washed sequentially (5 min per wash) on a rotating platform with 1 ml each of low salt washing buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl and 150 mM NaCl (pH 8.0)], high salt washing buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl and 500 mM NaCl (pH 8.0)], LiCl washing buffer [0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0)] and 1 \times TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)]. After the final wash, the pellet was eluted by resuspension in freshly made elution buffer (1% SDS and 50 mM NaHCO₃), followed by centrifugation. Twenty microliters of 5 M NaCl was added to the supernatant and the mixture incubated for 4 h at 65°C to reverse the protein-DNA cross-linking, and then the DNA was amplified using PCR. The cycling conditions were 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s.

Statistical analysis

The data were expressed as mean \pm standard error of the mean (SEM). Differences between groups were analyzed using one-way ANOVA in commercial statistical software (GraphPad Prism 4.02; GraphPad Software, San Diego, CA). Tukey's procedure was used to test differences between groups found significant using the *F*-test. Student's *t*-test was used to compare differences between normoxic and hypoxic groups. Statistical significance was set at $P < 0.05$.

RESULTS

Expression of CAD is cell-cycle-dependent

The expression of CAD provides a functional enzyme for the biosynthesis of pyrimidine nucleotides, the critical constituent for DNA synthesis and cell-cycle progression. To determine the expression levels of CAD in different cell types, mRNA encoding for CAD was quantified using real-time RT-PCR. The expression level of CAD was associated with the proliferation speed of each cancer cell line. Expression levels for the colo320DM and IMR32 lines (doubling time: ≤ 24 h) were significantly higher than for the primary cultured endometrial stromal cells (doubling time: > 48 h) (Figure 1). In addition, expression of CAD was cell-cycle-dependent. In colo320DM cells, CAD transcripts increased 4 h after serum stimulation, reached peak value at 8 h, returned to basal level at 16 h, and rose again at 24 h (Figure 1). Levels of CAD mRNA in IMR32 did not increase until 8 h after serum stimulation, maintained at the high value at 16 h, and returned to basal level at 24 h (Figure 1). In stromal cells, which showed no measurable proliferation within 24 h after serum stimulation, the CAD level predictably did not change (Figure 1).

Hypoxia inhibits the expression of CAD mRNA

In order to investigate whether the expression of CAD is regulated by hypoxia, we compared the mRNA levels of CAD in cells cultured under normoxic (21% O₂) or hypoxic (1% O₂) conditions. In colo320DM cells exposed to hypoxia,

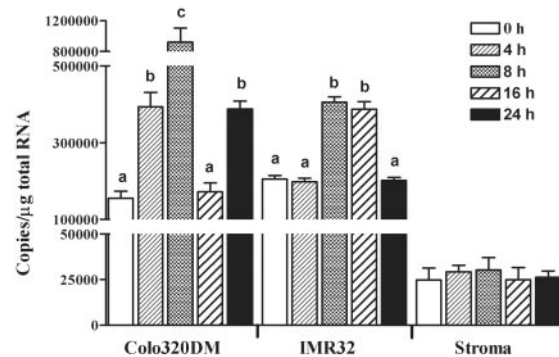


Figure 1. Expression of CAD mRNA in different cell types after serum stimulation. Cells were serum-starved for 24 h and then incubated in fresh medium containing 10% serum. Total RNA was isolated from cells at time points indicated and subjected to RT-PCR amplification. Concentrations of CAD transcripts were determined by real-time RT-PCR. Different letters indicate significant difference within each cell type ($P < 0.05$). Colo320DM: colon cancer cell line, IMR32: neuroblastoma cell line, stroma: primary cultured human uterine endometrial stromal cells.

CAD mRNA expression was significantly reduced at all time points (Figure 2A). To test whether hypoxia-induced suppression of CAD mRNA expression is a common effect, three other cell lines (A293T, IMR32 and HeLa) and human endometrial stromal cells were exposed to normoxia or hypoxia for 16 h, and their levels of mRNA were analyzed. Hypoxia treatment significantly inhibited CAD mRNA expression in all cells tested (Figure 2B), which indicated that it is a common phenomenon. It has been reported that hypoxia may induce apoptosis in p53^{+/+} cells (2). To test whether the decrease in CAD mRNA expression under hypoxia condition is due to hypoxia-induced cell death, cell numbers were calculated 16 h after hypoxia treatment. No significant decreases in cell numbers were observed in any of the cell lines used (data not shown). In addition, expression of another hypoxia-regulated gene, VEGF, was examined using simple RT-PCR as an internal control because hypoxia induces VEGF expression in many cell types and solid tumors. In fact, VEGF expression increased in cells exposed to hypoxia for 24 h even though CAD mRNA expression decreased (Figure 2C). Together, these

data demonstrate that hypoxia specifically suppressed CAD mRNA expression.

Suppression of CAD expression under chemical hypoxia

Hypoxia-regulated gene expression is either HIF-1-dependent or HIF-1-independent. To determine whether suppression of CAD expression under hypoxia is mediated by HIF-1, cells were treated with DFO, an iron chelator that causes HIF-1 α to accumulate. DFO treatment caused a dose-dependent increase in nuclear HIF-1 α (Figure 3A). A time-course experiment demonstrated that accumulation of HIF-1 α had discernibly accumulated in the nucleus 4 h after treatment in colo320DM cells, peaked at 8 h, and remained for at least 24 h (Figure 3A, D4, D8, D16, D24). Similarly, the expression of CAD was time-dependently suppressed after treatment with DFO under normoxic conditions (Figure 3B). Although the suppression pattern was somewhat different in different cell types, it seems clear that DFO reduced CAD expression in all four cell lines (colo320DM, HeLa, A293T and IMR32) as well as in the primary cultured stromal cells (Figure 3B). Again, the cell

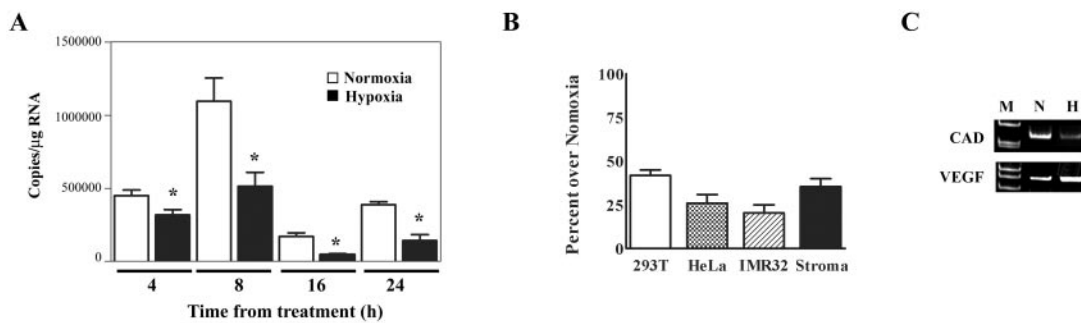


Figure 2. Hypoxia suppresses CAD mRNA expression. (A) Colo320DM cells were serum-starved for 24 h followed by addition of fresh medium supplemented with 10% FBS. Cells were then cultured in hypoxic chamber (hypoxia, 1% O₂) or regular incubator (normoxia, 21% O₂) for 4, 8, 16 and 24 h, respectively. Total RNA was isolated and levels of CAD transcripts were determined by real-time RT-PCR as described in experimental procedures. The data show means and standard errors obtained from three independent experiments performed in duplicate. Asterisk indicates significant difference (*P* < 0.05) compared to same time point normoxia group by unpaired *t*-test. (B) Serum-starved cells were cultured in fresh medium supplemented with 10% FBS under normoxia and hypoxia conditions, respectively for 16 h and expression of CAD mRNA was quantified. Data show mean values of hypoxia to normoxia ratio of three independent experiments. Three human cell lines (293T, HeLa and IMR32) and one primary culture human uterine endometrial stromal cell (stroma) were used. (C) Serum-starved Colo320DM cells were cultured in fresh medium supplemented with 10% FBS under normoxia and hypoxia conditions, respectively for 16 h and expression of CAD and VEGF mRNA was determined by simple RT-PCR.

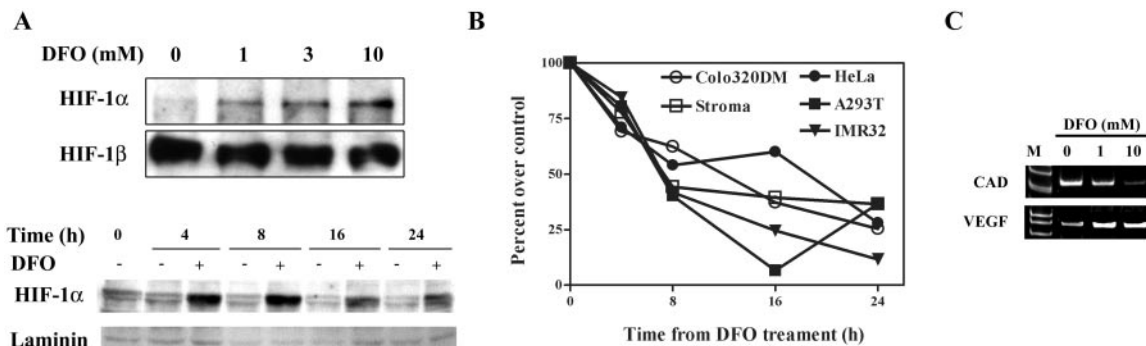


Figure 3. Hypoxia-suppressed CAD expression can be mimicked by DFO treatment. (A) Serum-starved colo320DM cells were cultured in fresh medium containing 10% FBS in the presence or absence of different concentrations of DFO and levels of nuclear HIF-1 α and HIF-1 β were determined (upper panel). Lower panel shows that nuclear HIF-1 α in cells treated with 10 mM DFO for different length of time as indicated. This experiment was repeated three times with similar result. (B) Serum-starved cells were cultured in fresh medium supplemented with 10% FBS in the presence or absence of 10 mM DFO for 0, 4, 8, 16 or 24 h. Expression of CAD transcripts were quantified by real-time RT-PCR. Data represent mean value of ratio to untreated control at the same time point (*n* = 3–4 for each cell types quantified in duplicate). (C) A representative gel picture shows RT-PCR products of CAD and VEGF after DFO treatment for 24 h.

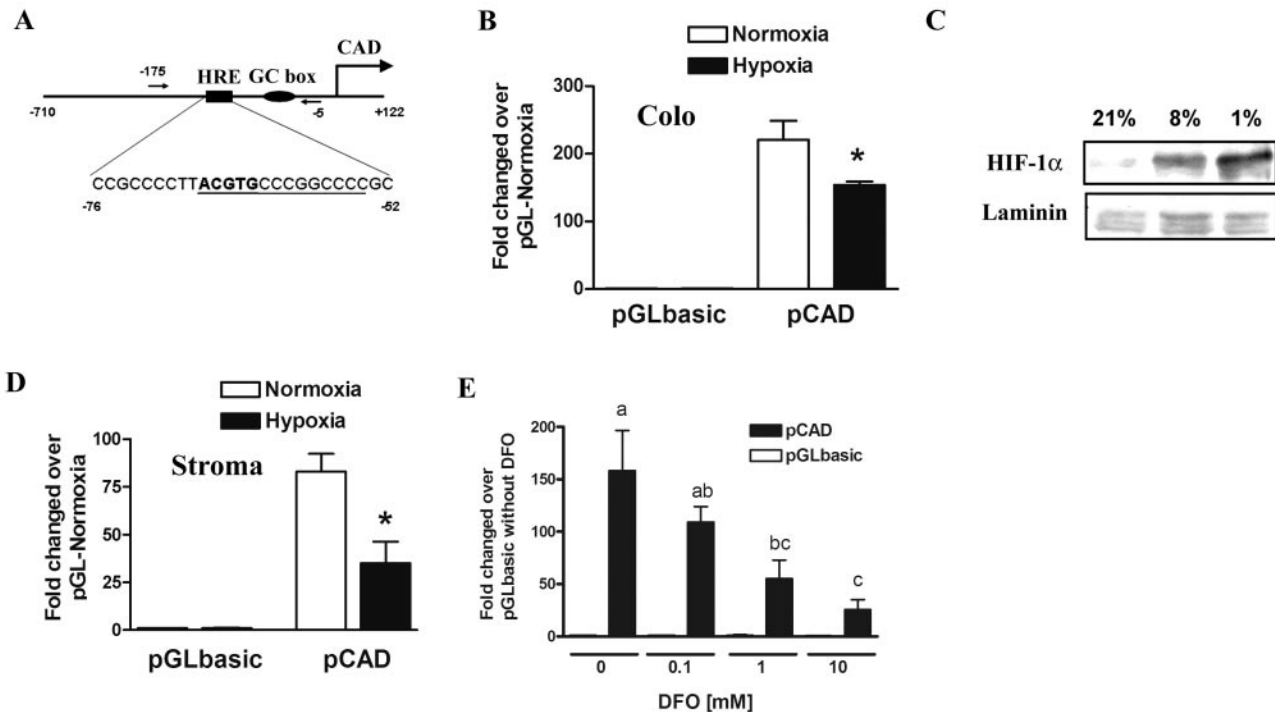


Figure 4. HIF-1 α suppressed promoter activity of human *cad* gene. (A) Schematic drawing of human *cad*-gene promoter shows location and sequences of bioinformatic predicted HRE matrix with core HRE in underlined bold face. Arrows indicate primers for chromatin immunoprecipitation-PCR assay. (B) Colo320DM cells was transfected with pGLbasic plasmid or pGL basic plasmid with human *cad* promoter (designated as pCAD) and subjected to normoxia (21% O₂) or hypoxia treatment (1% O₂) as described in experimental procedures. Data show means and standard errors of three independent experiments performed in duplicate. Asterisk indicates significant difference compared to normoxia group a $P < 0.05$. (C) A representative western blot result shows nuclear HIF-1 α and laminin in cells cultured under normoxia (21% O₂), 8% O₂, or 1% O₂ for 16 h. This experiment was repeated three times with similar result. (D) Primary culture human endometrial stromal cells were treated as described in B and human *cad*-promoter activity was determined. Data show means and standard errors of four independent experiments using different batches of cells performed in duplicate. Asterisk indicates significant difference compared to normoxia group at $P < 0.05$. (E) Transfected colo320DM cells were treated with different concentrations of DFO for 16 h and promoter activity was determined. Data show means and standard errors of three independent experiments performed in duplicate. Different letters indicate significant difference ($P < 0.05$).

number did not decrease (data not shown) and DFO upregulated the expression of VEGF mRNA (Figure 3C and data not shown), which indicated DFO did not induce apoptosis in these cells within 24 h.

Identification of functional HRE in human *cad*-gene promoter

Based on these data, we investigated whether repression of CAD by hypoxia could be regulated by HIF-1 at the transcriptional level. The sequence of *cad* promoter, available in the human genome database (Ensembl), was retrieved and analyzed for putative HRE sites using a hidden Markov model (HMM). An HRE matrix-containing the core sequence (5'-RCGTG-3') was identified at the -67/-54 of *cad* promoter (Figure 4A). To test whether this HRE matrix is functional, the 5'-flanking sequence of human *cad* (-710/+122) was cloned to a pGL3 basic luciferase reporter plasmid. Exposing cells transiently transfected with plasmid containing *cad* promoter to 1% oxygen significantly reduced reporter-gene expression (Figure 4B); the reduction was inversely correlated with the elevation of HIF-1 α in the nucleus (Figure 4C). This result was indisputably replicated in primary culture cells and three other cell lines (Figure 4D and data not shown) indicating, again, that it is a common event. Because DFO treatment also reduced CAD expression, we tested whether DFO suppressed

cad-promoter activity. As expected, treatment with DFO dose-dependently reduced reporter-gene expression in cells transfected with plasmids containing *cad* promoter (Figure 4E).

One study reported that the core HRE (5'-RCGTG-3') is necessary but not sufficient for HIF-mediated promoter activity (25). To test whether this HRE matrix is indeed a functional HIF-1-regulated region, two 25 bp oligonucleotides corresponding to the -76/-52 segment of the human *cad*-gene promoter region were synthesized and cloned to an SV40 promoter containing pGL3 plasmid (Figure 5A). Insertion of the HRE matrix into pGL3-SV40 plasmid caused a 2-fold increase in promoter activity when cells were cultured in normoxic conditions (Figure 5B). When exposed to hypoxia, reporter-gene expression decreased in cells transfected with this HRE-containing plasmid (Figure 5B).

To examine whether the effect of *cad* HRE was due to alteration of intact SV40 promoter, a 47 bp VEGF HRE (26) was cloned to the same position in the pGL3-SV40 plasmid, and luciferase activity was measured. The VEGF HRE did not alter promoter activity under normoxic conditions but it did induce reporter-gene expression under hypoxic conditions (Figure 5B). Similarly, cells transfected with plasmid containing the HRE matrix of the *cad* gene showed reduced promoter activity after being treated with 10 mM DFO (Figure 5C). In contrast, DFO treatment did not inhibit

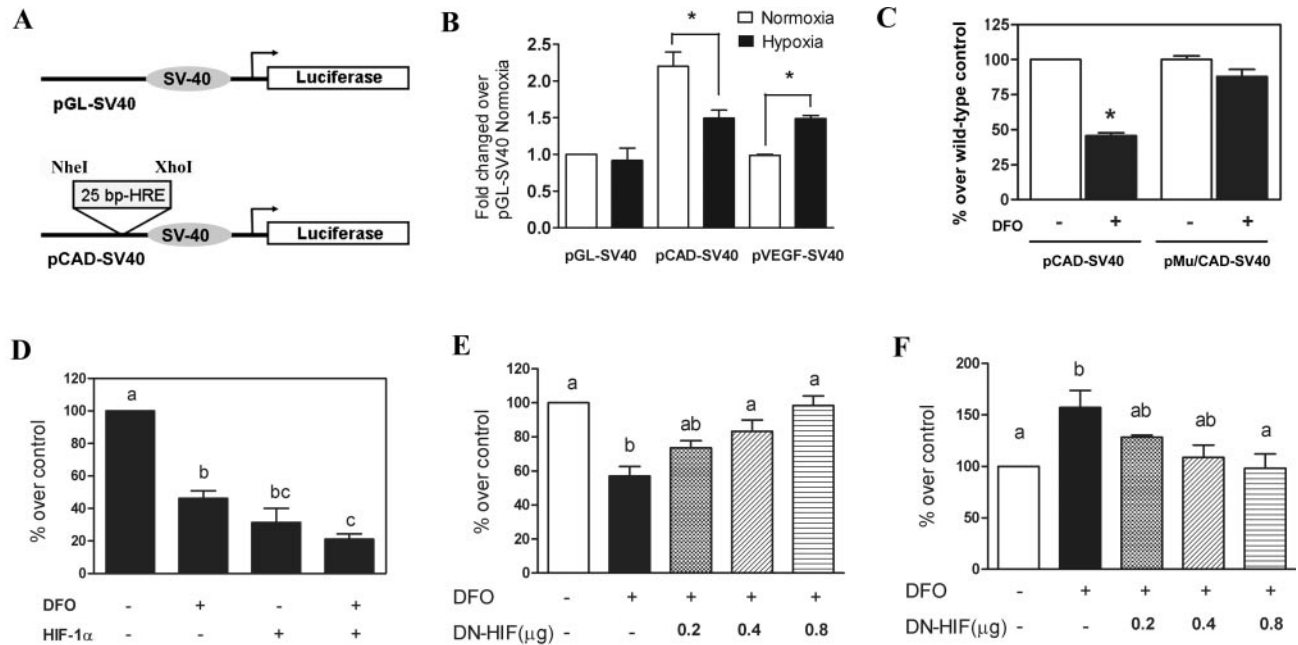


Figure 5. Effect of dominant-negative form HIF-1 α on hypoxia-regulated *cad*-promoter activity. (A) Schematic drawing of reporter constructs depicts that the 25-base HRE matrix of human *cad* gene was inserted between NheI and XhoI sites of pGL-SV40 vector. (B) Colo320DM cells were transfected with pGL-SV40 vector or HRE matrix-containing pCAD-SV40 plasmid and subjected to normoxia or hypoxia treatment as described in experimental procedure. As positive control, a 47-base VEGF HRE was also ligated to the same pGL-SV40 vector (termed pVEGF-SV40) and was used to perform identical experiment as described above side-by-side. Data show means and standard errors of three independent experiments performed in duplicate. Asterisk indicates significant difference from normoxia group by unpaired *t*-test at $P < 0.05$. (C) Cells were transfected with plasmids containing wild-type (pCAD-SV40) or mutated (pMuCAD-SV40) HRE as described. Transfected cells were then treated with 10 mM DFO for 16 h and luciferase activity was measured. Data show means and standard errors of three independent experiments performed in duplicate. Asterisk indicates significant difference from untreated group by unpaired *t*-test at $P < 0.05$. (D) Cells were co-transfected with pCAD and HIF-1 α or control vector and then treated with or without DFO (10 mM) for 16 h. Data show means and standard errors of three independent experiments performed in duplicate. Different letters indicate significant difference ($P < 0.05$). (E and F) Cells were co-transfected with pCAD-SV40 (E) or pVEGF-SV40 (F) and different amounts of dominant-negative HIF-1 α vector (DN-HIF) or control vector as indicated. Transfected cells were then treated with 10 mM DFO for 16 h and luciferase activity was measured. Data show means and standard errors of three independent experiments performed in duplicate. Different letters indicate significant difference ($P < 0.05$).

reporter-gene expression in cells transfected with plasmids containing mutated HRE matrix (Figure 5C). Forced expression of wild-type HIF-1 α significantly inhibited *cad*-promoter activity and this effect was further substantiated by DFO treatment (Figure 5D). Inversely, transfection with dominant-negative HIF-1 α plasmid dose-dependently restored *cad*-promoter activity inhibited by DFO treatment (Figure 5E). DFO treatment induced reporter-gene activity in cells co-transfected with pVEGF-SV40 and control plasmid, but this effect was abolished by overexpression of dominant-negative HIF-1 α (Figure 5F). Taken together, these data provide further evidence that supports the functional role of HIF-1 α in transcriptional repression of *cad*-gene activity in a gene-specific manner.

Suppression of *cad*-gene expression under hypoxia is HIF-1 α dependent

To determine the crucial role of HIF-1 α in hypoxia-mediated down-regulation of *cad*-gene expression, specific siRNA against HIF-1 α was used to knock down HIF-1 α and expression of CAD transcripts were determined under chemical hypoxia. Transfection of HIF-1 α siRNA (siHIF) significantly inhibited DFO-induced HIF-1 α protein accumulation while transfection of siRNA with scrambled sequence (sr-HIF) did not have similar effect (Figure 6A). Levels of

endogenous CAD transcripts were significantly decreased in mock transfected cells treated with DFO (Figure 6B). The suppressive effect was reversed by prior transfection with siHIF-1 α but not sr-HIF (Figure 6B) indicating suppression of CAD expression by DFO is HIF-1 α dependent.

Binding of HIF-1 α to the *cad*-promoter HRE

So far, we have identified that HIF-1 α transcriptionally inhibits *cad*-gene expression. To demonstrate that HIF-1 α physically binds to the *cad*-promoter HRE matrix and regulates *cad*-gene activity, colo320DM cells were exposed to normoxia or hypoxia for different periods of time, and the binding of HIF-1 α to *cad* promoter was analyzed using a ChIP assay. HIF-1 α bound significantly to the *cad* promoter by 16 h after hypoxia treatment (Figure 7A). In contrast, non-specific binding was unaffected by hypoxia treatment, as demonstrated by results using rabbit IgG as the primary antibody for immunoprecipitation (Figure 7A). Similar results were obtained when cells were treated with 1 mM DFO (Figure 7B). The binding of HIF-1 α to *cad* promoter was significantly increased by DFO treatment by as early as 4 h, and it remained so for up to 24 h (Figure 7B). In IMR32 neuroblastoma cells, basal binding of HIF-1 α to *cad* promoter under normoxia was greater, but DFO treatment also increased the binding (Figure 7B).

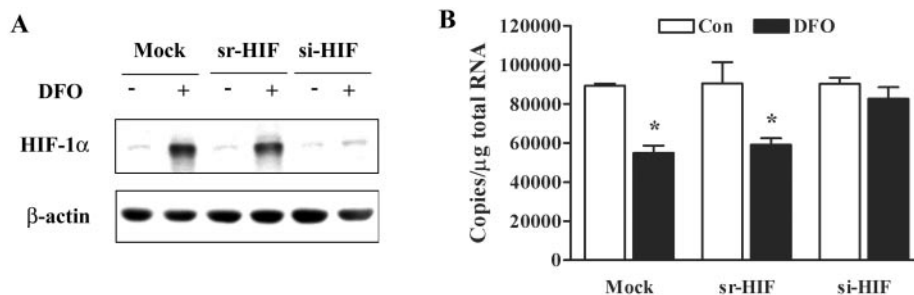


Figure 6. DFO-mediated suppression of CAD mRNA is HIF-1 α dependent. Cells were transfected with specific siRNA against HIF-1 α (siHIF), scrambled sequence of siRNA (sr-HIF), or transfection reagent only (Mock) as indicated. Transfected cells were then treated with 10 mM DFO or vehicle (Con) for 8 h and levels of endogenous HIF-1 α protein (A) and CAD mRNA transcripts (B) were determined by western blot and/or real-time RT-PCR. (A) a representative western blot shows that HIF-1 α was induced by DFO treatment in cells transfected with scrambled siRNA or mock transfection but not in cells transfected with siHIF-1 α . (B) Data show means and standard errors of three independent experiments performed in duplicate. Asterisks indicate significant difference from vehicle treated group ($P < 0.05$).

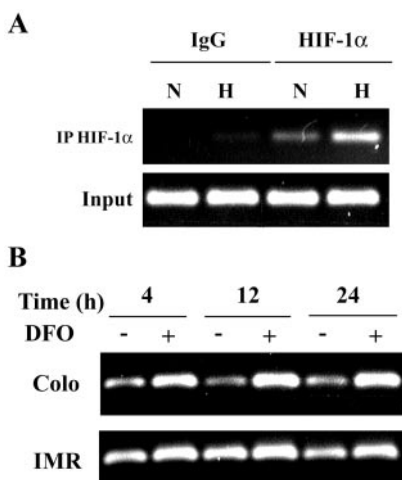


Figure 7. *In vivo* binding of HIF-1 α on the HRE matrix located at $-67/-54$ of the *cad* promoter. (A) Colo320DM cells were cultured under normoxia (N) or hypoxia (H) conditions for 16 h as described and subjected to chromatin immunoprecipitation-PCR amplification (ChIP) assay. Anti-HIF-1 α polyclonal antibody (HIF-1 α) or rabbit IgG (IgG) was used to precipitated sonicated chromatin. Sonicated cell lysate (2 μ l) was used as input control. (B) Colo320DM and IMR32 cells were treated with DFO (10 mM for Colo320DM and 1 mM for IMR32, respectively) for indicated time and ChIP assays were performed. V: DMSO vehicle treated groups, D: DFO-treated groups. Number indicates time of treatment in hour. The gel pictures are representative of four independent experiments.

DISCUSSION

Hypoxic stress inhibits *de novo* biosynthesis of pyrimidine nucleotides, thus leading to cell-cycle arrest due to an insufficient supply of building materials for DNA synthesis (7). Nevertheless, how hypoxia causes reduction in pyrimidine biosynthesis remains enigmatic. The present study provides evidence demonstrating that HIF-1 α transcriptionally inhibits the *cad* gene, which encodes a multifunctional enzyme that controls the rate-limiting step of pyrimidine biosynthesis (15,16). This conclusion is based on results obtained in experiments in which CAD expression declined in response to an increase in cellular levels of HIF-1 α . A functional HRE was identified in human *cad* promoter, which was bound by HIF-1 α as demonstrated by the ChIP assay. The use of dominant-negative form HIF-1 α totally abolished the hypoxic

effect provided further evidence to show the pivotal role of HIF-1 α in suppressing *cad*-promoter activity. Furthermore, increasing HIF-1 α levels in colo320DM, HeLa, A293T, IMR32 and primary endometrial stromal cells significantly inhibited *cad* expression, which unequivocally proved that repression of *cad*-promoter activity by HIF-1 α is common in hypoxic cells. Taken together, our current data provide evidence to support that hypoxia-induced cell-cycle arrest might be the result of the inhibition of CAD expression by HIF-1 α .

DNA synthesis diminishes when oxygen is reduced from cell culture (27), which ultimately leads to cell-cycle arrest at the G1/S-phase. Previous studies focused on two oxygen-dependent enzymes, ribonucleotide reductase and dihydroorotate dehydrogenase. They concluded by hypothesizing that inactivation of these enzymes under hypoxia is the primary cause of a shortage of deoxynucleotide precursors for DNA replication and a consequent arrest of cell-cycle progression (28,29). However, these two enzymes work downstream of CAD, and other studies (15,16) suggest that the rate-limiting reaction of the entire pyrimidine synthesis is actually controlled at the step of converting glutamine to dihydroorotate, which involves three enzymatic activities possessed by CAD. In agreement with these reports, our results demonstrated that the expression of CAD is highly associated with the cell-cycle progression and the expression level of CAD truly reflects the proliferation rate of cells. The highest level of CAD expression in colo320DM cells occurred between 4 and 8 h after serum stimulation, which demonstrates CAD's functional role of providing nucleotides for DNA replication. Furthermore, we found that oxygen deprivation or increased cellular HIF-1 α levels induced by DFO treatment significantly reduced the levels of CAD transcripts in colo320DM, HeLa, A293T, IMR32 and human endometrial stromal cells. These results not only demonstrate hypoxia-down-regulated CAD expression is a common phenomenon but also provide evidence to support the notion that hypoxia-induced cell-cycle arrest is directly related to the shortage of deoxynucleotide precursors for DNA synthesis (1).

The expression of hypoxia-responsive genes is primarily regulated by HIF-1 α / HIF-1 β transcription factor. The HIF heterodimer binds to a specific DNA-binding element, the HRE matrix, which contains a core sequence (5'-RCGTG-3') and uncharacterized flanking sequences in the promoter region

or other untranslated regions (30,31). Owing to the shortness of the sequence, the core HREs were observed frequently in the target-gene promoter, but only a few of them are effective for hypoxia-regulated gene expression. Cumulative data have demonstrated that the core HRE is necessary but not sufficient to regulate hypoxia-responsive genes (25,32,33). By using bioinformatic methodology, we identified one such HRE matrix in the proximal region of human *cad* promoter (−67/−54) and confirmed that this HRE matrix is important in HIF-1 α -regulated *cad*-promoter activity by providing several lines of evidence. First, the promoter activity of the intact human *cad* promoter (−710/+122) was down-regulated under hypoxic condition or an increase in nuclear HIF-1 α concentrations caused by DFO treatment. Second, the 25 bps human *cad* HRE matrix-containing plasmid responded to hypoxic stress exactly as that of intact *cad* promoter. Third, effect of hypoxia on *cad*-promoter activity was abrogated when the 25 bp HRE was mutated or by forced overexpression of dominant-negative HIF-1 α . Fourth, physical binding of HIF-1 α to the *cad* promoter was demonstrated by ChIP assay and the amounts of *cad* promoter-bound HIF-1 α was significantly increased under hypoxic conditions.

The repression of *cad*-promoter activity by HIF-1 α concurs with the notion that hypoxia causes a halt of pyrimidine nucleotide biosynthesis and that HIF-1 α is indispensable for hypoxia-induced cell-cycle arrest (4). However, the in-depth mechanism responsible for inhibiting HIF-1-mediated *cad*-gene expression is not completely elucidated. The proximal *cad* promoter contains an E-box essential for c-Myc-induced CAD expression (34). It has been reported that HIF-1 α can counteract c-Myc's function independent of HIF-1 α 's transcriptional activity and DNA-binding ability (35). In the current study, by showing that dominant-negative HIF-1 α blocked the effects of hypoxia, we found that the binding of HIF-1 α to the HRE matrix and retention of transcriptional capability are necessary for suppression of *cad*-promoter activity. Alternatively, it was reported that ATP-dependent hSWI/SNF chromatin remodeling complex containing mSin3A/histone deacetylase 2 (HDAC2) co-repressor might also be involved in the transcriptional repression of the *cad* gene (36). It is possible that the binding of HIF-1 α to the HRE matrix recruits histone modification complex or other co-repressors to *cad* promoter and thus suppresses gene transcription. Further investigations on the nature of the partners of HIF-1 involved in *cad* down-regulation are crucial to elucidate the mechanisms involved in the repression of HIF-1-dependent hypoxia down-regulated genes.

In summary, we have demonstrated for the first time that HIF-1 α transcriptionally suppressed *cad*-gene expression under hypoxia. This effect was unequivocally observed in four cancerous and non-cancerous cell lines and one primary culture cell type, which demonstrated that it is a common phenomenon. Given the critical role of CAD in nucleotides biosynthesis and consequent DNA replication, our data provide a functional link between hypoxia and cell-cycle arrest. Further investigation is warranted to explore effects of CAD suppression on the upregulation of checkpoint proteins and cell-cycle arrest when cells are under hypoxic stress, which may provide valuable information in designing novel regimens for treating hypoxia-related diseases.

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REFERENCES

- Ameltem, O., Sandvik, J.A., Stokke, T. and Pettersen, E.O. (1998) The retinoblastoma protein-associated cell cycle arrest in S-phase under moderate hypoxia is disrupted in cells expressing HPV18 E7 oncoprotein. *Br. J. Cancer*, **77**, 862–872.
- Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W. and Giaccia, A.J. (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature*, **379**, 88–91.
- Schmaltz, C., Hardenbergh, P.H., Wells, A. and Fisher, D.E. (1998) Regulation of proliferation-survival decisions during tumor cell hypoxia. *Mol. Cell Biol.*, **18**, 2845–2854.
- Goda, N., Ryan, H.E., Khadivi, B., McNulty, W., Rickert, R.C. and Johnson, R.S. (2003) Hypoxia-inducible factor 1 α is essential for cell cycle arrest during hypoxia. *Mol. Cell Biol.*, **23**, 359–369.
- Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P. *et al.* (1998) Role of HIF-1 α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature*, **394**, 485–490.
- Gardner, L.B., Li, Q., Park, M.S., Flanagan, W.M., Semenza, G.L. and Dang, C.V. (2001) Hypoxia inhibits G1/S transition through regulation of p27 expression. *J. Biol. Chem.*, **276**, 7919–7926.
- Löffler, M. (1989) The biosynthetic pathway of pyrimidine (deoxy)nucleotides: a sensor of oxygen tension necessary for maintaining cell proliferation? *Exp. Cell Res.*, **182**, 673–680.
- Green, S.L., Freiberg, R.A. and Giaccia, A.J. (2001) p21(Cip1) and p27(Kip1) regulate cell cycle reentry after hypoxic stress but are not necessary for hypoxia-induced arrest. *Mol. Cell Biol.*, **21**, 1196–1206.
- Semenza, G.L. (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol.*, **88**, 1474–1480.
- Wiener, C.M., Booth, G. and Semenza, G.L. (1996) *In vivo* expression of mRNAs encoding hypoxia-inducible factor 1. *Biochem. Biophys. Res. Commun.*, **225**, 485–488.
- Tian, H., McKnight, S.L. and Russell, D.W. (1997) Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.*, **11**, 72–82.
- Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y. and Fujii-Kuriyama, Y. (1997) A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1 α regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc. Natl Acad. Sci. USA*, **94**, 4273–4278.
- Flamme, I., Frolich, T. and Risau, W. (1997) Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J. Cell Physiol.*, **173**, 206–210.
- Graeber, T.G., Peterson, J.F., Tsai, M., Monica, K., Fornace, A.J., Jr and Giaccia, A.J. (1994) Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol. Cell Biol.*, **14**, 6264–6277.
- Coleman, P.F., Suttle, D.P. and Stark, G.R. (1977) Purification from hamster cells of the multifunctional protein that initiates *de novo* synthesis of pyrimidine nucleotides. *J. Biol. Chem.*, **252**, 6379–6385.
- Iwahana, H., Fujimura, M., Ii, S., Kondo, M., Moritani, M., Takahashi, Y., Yamaoka, T., Yoshimoto, K. and Itakura, M. (1996) Molecular cloning of a human cDNA encoding a trifunctional enzyme of carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase in *de Novo* pyrimidine synthesis. *Biochem. Biophys. Res. Commun.*, **219**, 249–255.
- Jones, M.E. (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. *Annu. Rev. Biochem.*, **49**, 253–279.

18. Graves, L.M., Guy, H.I., Kozlowski, P., Huang, M., Lazarowski, E., Pope, R.M., Collins, M.A., Dahlstrand, E.N., Earp, H.S., 3rd and Evans, D.R. (2000) Regulation of carbamoyl phosphate synthetase by MAP kinase. *Nature*, **403**, 328–332.
19. Khan, S., Abdelrahim, M., Samudio, I. and Safe, S. (2003) Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17beta-estradiol in breast cancer cells. *Endocrinology*, **144**, 2325–2335.
20. Loffler, M. (1985) Towards a further understanding of the growth-inhibiting action of oxygen deficiency. Evaluation of the effect of antimycin on proliferating Ehrlich ascites tumour cells. *Exp. Cell Res.*, **157**, 195–206.
21. Loffler, M. (1985) Characterization of the deoxynucleoside-dependent reversal of hypoxia-induced inhibition of cell cycle progression in Ehrlich ascites tumor cells. *Eur. J. Cell Biol.*, **39**, 198–204.
22. Tsai, S.J., Wu, M.H., Lin, C.C., Sun, H.S. and Chen, S.M. (2001) Regulation of steroidogenic acute regulatory protein expression and progesterone production in endometriotic stromal cells. *J. Clin. Endocrinol. Metab.*, **86**, 5765–5773.
23. Tsai, S.J., Wu, M.H., Chen, H.M., Chuang, P.C. and Wing, L.Y. (2002) Fibroblast growth factor-9 is an endometrial stromal growth factor. *Endocrinology*, **143**, 2715–2721.
24. Sun, H.S., Hsiao, K.Y., Hsu, C.C., Wu, M.H. and Tsai, S.J. (2003) Transactivation of steroidogenic acute regulatory protein in human endometriotic stromal cells is mediated by the prostaglandin EP2 receptor. *Endocrinology*, **144**, 3934–3942.
25. Wang, G.L., Jiang, B.H., Rue, E.A. and Semenza, G.L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc. Natl Acad. Sci. USA*, **92**, 5510–5514.
26. Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D. and Semenza, G.L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell Biol.*, **16**, 4604–4613.
27. Balin, A.K., Fisher, A.J. and Carter, D.M. (1984) Oxygen modulates growth of human cells at physiologic partial pressures. *J. Exp. Med.*, **160**, 152–166.
28. Chimpoy, K., Tassotto, M.L. and Mathews, C.K. (2000) Ribonucleotide reductase, a possible agent in deoxyribonucleotide pool asymmetries induced by hypoxia. *J. Biol. Chem.*, **275**, 39267–39271.
29. Amellem, O., Loffler, M. and Pettersen, E.O. (1994) Regulation of cell proliferation under extreme and moderate hypoxia: the role of pyrimidine (deoxy)nucleotides. *Br. J. Cancer*, **70**, 857–866.
30. Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N. and Fujii-Kuriyama, Y. (1995) Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. *Proc. Natl Acad. Sci. USA*, **92**, 1936–1940.
31. Chapman-Smith, A., Lutwyche, J.K. and Whitelaw, M.L. (2003) Contribution of the PAS domains to DNA binding by the basic helix-loop-helix PAS transcriptional regulators. *J. Biol. Chem.*, **279**, 5353–5362.
32. Swanson, H.I., Chan, W.K. and Bradfield, C.A. (1995) DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J. Biol. Chem.*, **270**, 26292–26302.
33. Wood, S.M., Gleadle, J.M., Pugh, C.W., Hankinson, O. and Ratcliffe, P.J. (1996) The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *J. Biol. Chem.*, **271**, 15117–15123.
34. Boyd, K.E., Wells, J., Gutman, J., Bartley, S.M. and Farnham, P.J. (1998) c-Myc target gene specificity is determined by a post-DNA binding mechanism. *Proc. Natl Acad. Sci. USA*, **95**, 13887–13892.
35. Koshiji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C. and Huang, L.E. (2004) HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *EMBO J.*, **23**, 1949–1956.
36. Pal, S., Yun, R., Datta, A., Lacomis, L., Erdjument-Bromage, H., Kumar, J., Tempst, P. and Sif, S. (2003) mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the myc target gene cad. *Mol. Cell Biol.*, **23**, 7475–7487.