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Transcriptional Up-Regulation of FoxM1 in Response to Hypoxia Is Mediated by HIF-1

Li-Min Xia,[1] Wen-Jie Huang,[2] Bo Wang,[1] Mei Liu,[1] Qiong Zhang,[1] Wei Yan,[1] Qian Zhu,[1] Min Luo,[1] Zhen-Zhen Zhou,[1] and De-An Tian[1*]

[1] *Division of Gastroenterology, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China*

[2] *State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China*

ABSTRACT

The proliferation-specific Forkhead box M1 (FoxM1) transcription factor is overexpressed in cancer cells and acts as an important regulator of cancer cell growth and survival. Here, we show the molecular mechanisms by which hypoxia regulate FoxM1 expression in cancer cells. When cells were subjected to hypoxia (1% O_2), the mRNA and protein levels of FoxM1 had a significant increase in cancer cells (HepG2, MCF-7, and HeLa). Such increase was due to the direct binding of hypoxia-inducible factor 1 (HIF-1) to the HIF-1 binding sites in the FoxM1 promoter. By deletion and mutation assays, we demonstrated that the HIF1-1 and HIF1-3/4 binding sites on the FoxM1 promoter were essential for transcriptional activation of FoxM1 by hypoxia. We also demonstrated that HIF-1α directly bound to the promoter of FoxM1 and the binding was specific, as revealed by HIF-1 binding/competition assay and chromatin immunoprecipitation assay. Consequently, the up-regulation of FoxM1 accelerated the growth of hypoxic cancer cells by decreasing nuclear levels of p21 and increasing expression of cyclin B1 and cyclin D1. These findings provide a new insight into how tumor cells overcome hypoxic stress and survive, and also disclose a new regulatory mechanism of FoxM1 expression in tumor cells. J. Cell. Biochem. 106: 247–256, 2009.

KEY WORDS: FOXM1; HYPOXIA; HIF-1; PROMOTER

Hypoxia, which refers to oxygen deficiency in tissues, is a universal hallmark of solid tumors and it represents a key regulatory factor in tumor growth and survival [Harris, 2002]. Recent studies showed that tumor cells adapt to hypoxic microenvironment by regulating several genes involved in angiogenesis, cell proliferation, glucose metabolism, and cell survival [Giaccia et al., 2004]. These effects of hypoxia facilitate the survival of tumor cells and render tumor cells resistant to various cancer therapies, such as radiotherapy and chemotherapy [Kim et al., 2004].

The transcription factor HIF-1 functions as a global regulator of O_2 homeostasis and the adaptation to O_2 deprivation [Semenza, 2000]. HIF-1 is a heterodimer comprising a constitutively expressed HIF-1β subunit and a highly regulated HIF-1α subunit [Wang et al., 1995]. Classically, the abundance of HIF-1α protein is regulated by the HIF prolyl hydroxylases (PHDs) [Jiang et al., 1997] and the HIF asparaginyl hydroxylase factor-inhibiting HIF (FIH-1) [Mahon et al., 2001] in normoxic conditions. Under normoxic conditions, specific proline residues in the HIF-1α protein are hydroxylated by PHDs. The binding of the von Hippel Lindau tumor suppressor protein (pVHL), resulting in polyubiquitylation and rapid degradation [Isaacs et al., 2002]. Similarly, a conserved asparagine residue in the carboxyl-terminal transactivation domain (CAD) of the HIF-1α is hydroxylated at normoxia by FIH-1, preventing the recruitment of the p300/CBP transcriptional coactivators leading to transcriptional repression [Mahon et al., 2001]. However, under hypoxic conditions, proline hydroxylation is inhibited, thereby stabilizing HIF-1α, which can translocate into the nucleus and bind to HIF-1β, forming the active HIF-1 complex [Jiang et al., 1997]. The HIF-1 complex can recruit other transcriptional factors and coactivators to transactivate a variety of genes containing the hypoxia-responsive elements [Gray et al., 2005].

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*Correspondence to: Prof. Dr. De-An Tian, Division of Gastroenterology, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China.
E-mail: datian@tjh.tjmu.edu.cn

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The mammalian forkhead box (Fox) family of transcription factors consists of more than 50 mammalian proteins [Carlsson and Mahlapuu, 2002] that share homology in the winged helix DNA-binding domain [Marsden et al., 1998]. The FoxM1 protein (previously known as HFH-11, Trident, WIN, MPP2) protein is a proliferation-specific member of the Fox family of transcription factors. Expression of FoxM1 is ubiquitous in all proliferating mammalian cells, and its expression is induced during the G1 phase of the cell cycle and continues during S-phase and mitosis [Yao et al., 1997; Ye et al., 1999]. Liver regeneration studies with mice in which the albumin promoter-enhancer Cre recombinase (Alb-Cre)-mediated conditional deletion of the FoxM1 LoxP/LoxP (fl/fl) targeted allele in adult hepatocytes showed that FoxM1 is required for hepatocyte DNA replication and mitosis [Wang et al., 2002a; Costa et al., 2003]. The FoxM1 protein decreases nuclear levels of the cdk inhibitor proteins p27 and p21 [Wang et al., 2002b; Wang et al., 2005], which promote cell cycle arrest by inhibiting activation of the cdk2/cyclin complexes. Furthermore, FoxM1 has been shown to regulate transcription of cell cycle genes essential for G1-S and G2-M progression and chromosome stability and segregation [Laoukili et al., 2005; Wonsey and Follettie, 2005].

Consistent with the important role in cell cycle progression, FoxM1 is found to be overexpressed in a variety of human tumors [Perou et al., 2000; Okabe et al., 2001; Kim et al., 2006]. In addition, the expression levels of FoxM1 increase with tumor grade and are inversely correlated with patient survival [Liu et al., 2006]. It is observed that a $p19^{ARF}$ peptide fragment physically interacts with FoxM1, suppresses FoxM1 transcriptional activity, and inhibits FoxM1-enhanced anchorage-independent growth [Kalinichenko et al., 2004]. These results suggest that FoxM1 may be an important regulator of cancer cell growth and survival. However, the molecular mechanisms of regulation FoxM1 expression in tumorigenic cells were unclear. In this study, we have examined the possibility that hypoxia regulated FoxM1 expression in cancer cells and explored the mechanisms involved.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

HepG2 hepatocellular carcinoma, MCF-7 breast carcinoma, and HeLa cervix carcinoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. For hypoxia experiments (1% O_2), subconfluent cells were incubated in serum-free CO_2-independent medium (Invitrogen) supplemented with 1 mM glutamine. YC-1 was purchased from Sigma–Aldrich Corporation, resuspended in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mg/ml, and stored at −20°C. The hypoxia surrogate deferoxamine and $CoCl_2$ were also purchased from Sigma–Aldrich Corporation.

RNA EXTRACTION AND QUANTITATIVE RT-PCR

The total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. RNase-free DNase (Promega) was used to eliminate the contamination of genomic DNA. cDNA was synthesized as described previously [Huang et al., 2006]. The following reaction mixture was used for all QRT-PCR samples: 1× of IQ SybrGreen Supermix (Bio-Rad), 200 nmol/L of each primer, and 2.5 μl of cDNA in a 25 μl total volume. Reactions were amplified and analyzed for three times using a ABI 7000 system (Applied-Biosystems). FoxM1 primers were 5′-TCTCAGCACCACTCCCTTG-3′ and 5′-GGATCTTGCTGAGGCTGTC-3′. Primers 5′-CTAACGTGT-TATCTGTCGCT-3′ and 5′-GGTTTCTGCTGCCTTGTAT-3′ were used to amply HIF-1α. β-actin was used as an internal control.

CYTOPLASMIC AND NUCLEAR PROTEIN EXTRACTION

For cytoplasmic extracts, cells were collected and incubated for 20 min at 4°C with lysis buffer [20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiotheitol, 10 mM NaCl, protease inhibitor cocktail tablet (Roche, Germany)], centrifuged at 3,000*g* for 5 min at 4°C and the supernatant retained. For nuclear extracts, the pellets were resuspended in lysis buffer containing 500 mM NaCl and gently rocked for 1.5 h at 4°C. The lysates were then centrifuged at 25,000*g* for 15 min at 4°C and the supernatant containing the nuclear proteins collected. Protein concentration was determined using the Bradford method.

WESTERN BLOT ASSAY

Cells were lysed in lysis buffer (50 mM Tris, pH7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, and protease inhibitor cocktail tablet) for 15 min or ice followed by centrifugation at 12,000*g* for 20 min, protein concentration was determined using the Bradford method. Equal amounts of sample lysates were separated by running through 8–12% SDS–PAGE gel and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with 5% milk in TBST (120 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. Blots were incubated with anti-HIF-1α (Sigma–Aldrich Corporation), anti-FoxM1, anti-β-actin, anti-p21, anti-Cyclin B1, or anti-cyclin D1 (Santa Cruz) antibody overnight at 4°C. The membranes were washed three times and incubated with HRP-conjugated secondary antibody. Proteins were visualized by Dura SuperSignal Substrate (Pierce).

PLASMID CONSTRUCTION

A 1038-bp FoxM1 promoter construct (−1012/+26)FoxM1, corresponding to the sequence from −1012 to +26 (relative to the transcriptional start site) of the 5′-flanking region of the human FoxM1 gene, was generated from human genomic DNA using F1 (5′-TGTTGGTACCGCAGTGGTGTGATCATGGCT-3′) and R1 (5′-TGTTAAGCTTGGGGTCTGGCACCGGAG-3′) for forward and reverse primers, respectively. The polymerase chain reaction product was cloned into *Kpn*I and *Hin*dIII sites of pGL3-Basic vector. The resulting construct was confirmed by DNA sequencing. The 5′-flanking deletion constructs of the FoxM1 promoter, (−600/+26)FoxM1, (−330/+26)FoxM1, (−178/+26)FoxM1, and (−58/+26)FoxM1 were similarly generated by using the (−1012/+26)FoxM1 construct as a template and the forward primes were F2 (5′-TGTTGGTACCTATCCGAAGGCTTGGCT-3′), F3 (5′-TGTTGG-TACCAGCGCGGCAGGAAAAGC-3′), F4 (5′-TGTTGGTACCTGCG-GTCCGCCTTA-3′), and F5 (5′-TCTTGGTACCTTAACGCTCCGCCG-

3′). Site-directed mutagenesis of four HIF-1 binding sites were done by multiple rounds of PCR using (−330/+26)FoxM1 construct and appropriate primers with altered bases. The primers used to make HIF1-1 mutant were: pair 1, F3, and R2 (5′-ACGCGCGGAAGCGGtagaACAGGCC-3′); pair 2, F6 (5′-GGCCTGTtctaCCGCTTCCGCGCGT-3′), and R1. For mutanting the HIF1-2 site, the primers used were: F7 (5′-CTCGCCCACCtctaCGGCGGGGACC-3′) and R3 (5′-GGTCCCCGCCGtagaGGTGGGCGAG-3′). For mutanting the HIF1-3/4 site, the primers used were: F8 (5′-CGTTCCGTCAtagaACCTTAACGCT-3′) and R4 (5′-AGCGTTAAGGTtctaTGACGGAACG-3′). The first two rounds of PCR generated two fragments of DNA, with 25 bp of overlap. These two fragments were gel purified and used as the templates for a third PCR with F3 and R1 primers. The mutated DNA was then cloned in pGL3-Basic and verified by sequence analysis.

TRANSIENT TRANSFECTION AND LUCIFERASE ACTIVITY MEASUREMENT

Each promoter construct was cotransfected with the pRL-TK plasmid into subconfluent (80–90%) monolayer cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 5 h of transfection, cells were washed and allowed to recover overnight in fresh medium. Twelve hours post-transfection, cells were incubated under normoxia or hypoxia for 12 h. Luciferase assays were detected with the Dual Luciferase Assay (Promega) according the manufacturer's instructions. The green fluorescent protein (GFP) control siRNA was 5′-CAAGCUGACCCUGAAGUUdTdT-3′.

HIF-1α BINDING AND COMPETITION ASSAY

HIF-1α binding and competition assay was done with a TransAM kit (Active Motif) according to the manufacturer's instructions. The kit measures binding to HIF-1 oligonucleotides from the EPO gene attached to a 96-well plate. Briefly, nuclear extracts were prepared from cells following exposure to normoxia or hypoxia for 24 h. To examine HIF-1α activation during hypoxia, 2, 5, and 10 μg of nuclear extracts were added to well. After incubation for 1 h, an anti-HIF-1α antibody and a horseradish peroxidase-conjugated secondary antibody were added, and the absorbance was then measured at 450 nm using Microplate Reader Model 680 (Bio-Rad). For competition assay, oligonucleotides containing wild-type and mutated HIF-1 binding oligonucleotides were synthesized and annealed to obtain double-stranded DNA fragments. The wild-type oligonucleotide sequence (the sequence of the sense strand was showed) was as follows: 5′-GGCCTGTCACGCCGCTTCCGCG-3′ (HIF1-1), 5′-TCGCCCACCCACGCGGCGGGGA-3′ (HIF1-2), and 5′-TTCCGTCACGTGACCTTAACGCT-3′ (HIF1-3/4). Its corresponding mutant sequence was 5′-GGCCTGTctaCCGCTTCCGCG-3′, 5′-TCGCCCACCtctaCGGCGGGGA-3′ and 5′-TTCCGTCAtagaACCTTAACGCT-3′. The wild-type or the mutant oligonucleotides of the EPO promoter or the FoxM1 promoter was first added to the appropriate well before addition of 10 μg of the nuclear extracts.

CHROMATIN IMMUNOPRECIPITATION ASSAY (CHIP)

Cells growing in T25 flasks under normoxia or hypoxia were cross-linked using 1% formaldehyde at 37°C for 10 min. After washing with ice cold PBS, cells were collected and resuspended for 10 min in 300 μl lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS,and protease inhibitor cocktail tablet). DNA was sheared to small fragments by sonication. The supernatant was precleared using herring sperm DNA/protein G-Sepharose slurry (Sigma–Aldrich Corporation). The recovered supernatant was incubated with either anti-HIF-1α antibody or an isotype control IgG for 2 h in the presence of herring sperm DNA and protein G-Sepharose beads. The immunoprecipitated DNA was retrieved from the beads with 1% SDS and 1.1 M $NaHCO_3$ solution at 65°C for 6 h. DNA was then purified using a PCR purification kit (Qiagen), and PCR was done on the extracted DNA using FoxM1 promoter-specific primers. The primers 5′-GGAGCAGAGGAGCCTGAG-3′ and 5′-GCGAGCCGAGGGAGAGTT-3′ were used for amplifying HIF1-1 binding site. The primers 5′-CGGAATGCCGAGACAAGG-3′ and 5′-TGGCACCGGAGCTTTCAGTT-3′ were used for HIF1-3/4 binding site.

CELL GROWTH ASSAY

For MTT assay, cells were seeded in 96-well plates at a density of 5×10^3 cells per well and incubated for 18 h. After transfection, cells were exposed to hypoxic conditions for 4 days. At indicated time, 50 μl MTT solution (2 mg/1 ml PBS) was added to each well, and the cells were further incubated at 37°C for 4 h. At the end of incubation period, the medium was discarded, washed with PBS for two times, added 200 μl DMSO/well to 96-well plate to solubilize formazan crystals. The absorbance was then measured at 570 nm using Microplate Reader Model 680 (Bio-Rad).

STATISTICAL ANALYSIS

Data were analyzed using the Statistical Package for Social sciences (SPSS) software (Version 11.0). Student's *t*-test was used and $P < 0.05$ was considered statistically significant.

RESULTS

THE EXPRESSION LEVELS OF FOXM1 ARE UP-REGULATED IN RESPONSE TO HYPOXIA

Since hypoxia is a common feature of the microenvironment of solid tumors, it is important to detect whether hypoxia promotes the expression of FoxM1 in tumor cells. To study the effect of hypoxia on FoxM1 expression in cancer cells, we exposed tumorigenic cells (HepG2, MCF-7, and HeLa) to hypoxic conditions (1% O_2). The expression levels of FoxM1 were determined by qRT-PCR and Western blot in the cells exposed to hypoxia for 0, 4, 8, and 24 h. As shown in Figure 1A, hypoxic exposure for 8 h strongly increased mRNA levels of FoxM1 in tumorigenic cells compared with the cells at normoxia (20% O_2), and the mRNA levels of FoxM1 increased in a time-dependent manner. Western blot assay showed that hypoxic exposure strongly increased protein levels of FoxM1 in tumorigenic cells (HepG2, MCF-7, and HeLa), and the protein levels of FoxM1 also increased in a time-dependent manner (Fig. 1B,C). Furthermore, we evaluated HIF-1α expression at the mRNA and protein levels. Consistent with other studies [Rivard et al., 2000], there was no difference of HIF-1α mRNA levels between the cells under hypoxic or normoxic conditions, while HIF-1α protein levels were up-regulated after the cells were exposed to hypoxia (Fig. 1B,C).

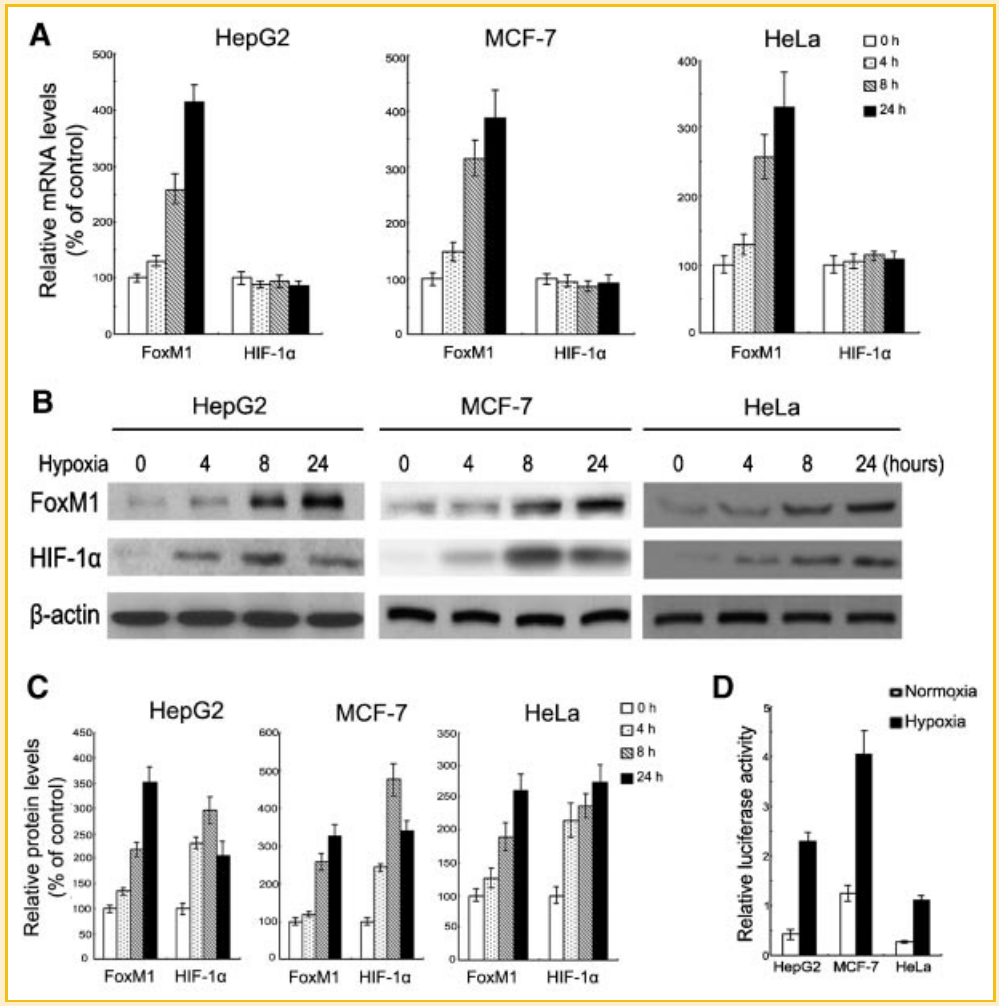

Fig. 1. Up-regulation of FoxM1 expression in cancer cells during hypoxia. A: HepG2, MCF-7, and HeLa cells were exposed to hypoxia for different time, and the mRNA levels of HIF-1α and FoxM1 were analyzed by qRT-PCR assay. β-actin was used as an internal control. The mRNA levels of HIF-1α and FoxM1 in normoxic cells were set to 100%, respectively. $^{*}P < 0.05$ versus control. B,C: The tumorigenic cells (HepG2, MCF-7, and HeLa) were exposed to hypoxia, and the protein levels of HIF-1α and FoxM1 were analyzed by Western blot assay. β-actin was used as an internal control. Densitometric analysis of HIF-1α and FoxM1 protein levels was performed using Quantity One software (Bio-Rad). The data were expressed as the mean ± SD and presented as percent amount of HIF-1α and FoxM1 (the expression levels of HIF-1α and FoxM1 in normoxic cells were set to 100%, respectively). The data represent three independent experiments. D: Indicated cells were transiently transfected with the FoxM1 promoter luciferase reporter and pRL-TK plasmids and grown in either normoxia or hypoxia for 24 h. The luciferase activity was determined. The data represented means of duplicate determinations in one representative experiment of three. $^{*}P < 0.05$ versus control.

Similar findings were made in tumorigenic cells (HepG2, MCF-7, and HeLa), which were treated with $CoCl_2$ and deferoxamine (100 μM each), respectively (Fig. 2A). $CoCl_2$ and deferoxamine are thought to activate hypoxia-induced genes at normal oxygen tension through deprivation of Fe^{2+}, which is a critical cofactor for HIF-1α prolyl hydroxylase, the rate-limiting enzyme in oxygen-dependent degradation of HIF-1α. Notably, stimulation of FoxM1 in HepG2, MCF-7, and HeLa cells by $CoCl_2$, and deferoxamine was associated with a marked increase in HIF-1α protein levels while β-actin content remained unaffected (Figs. 1 and 2).

FOXM1 IS TRANSCRIPTIONALLY UP-REGULATED DURING HYPOXIA

To further investigate the mechanism of FoxM1 up-regulation during hypoxia, we used a luciferase reporter assay to assess the FoxM1 promoter activity. The reporter plasmid was transfected into indicated cells and maintained overnight in fresh medium. Twelve hours post-transfection, cells were incubated under normoxia or hypoxia for 24 h. To correct for variable transfection efficiency, cells were cotransfected with pRL-TK. Cell extracts were assayed for Luc and Renilla activity. As shown in Figure 1D, exposure of cancer cells to hypoxia for 24 h resulted in significant increases in FoxM1 promoter activity compared with normoxia, suggesting that hypoxia may enhance FoxM1 transcription activity.

FOXM1 ACTIVITY IS INDUCED BY HYPOXIA IN A HIF-1–DEPENDENT MANNER

Because FoxM1 was transcriptionally up-regulated in hypoxic cancer cells and given the critical role that HIF-1 played in the cellular transcriptional response to hypoxia, we investigated whether FoxM1 induction was regulated by HIF-1. HepG2 cells

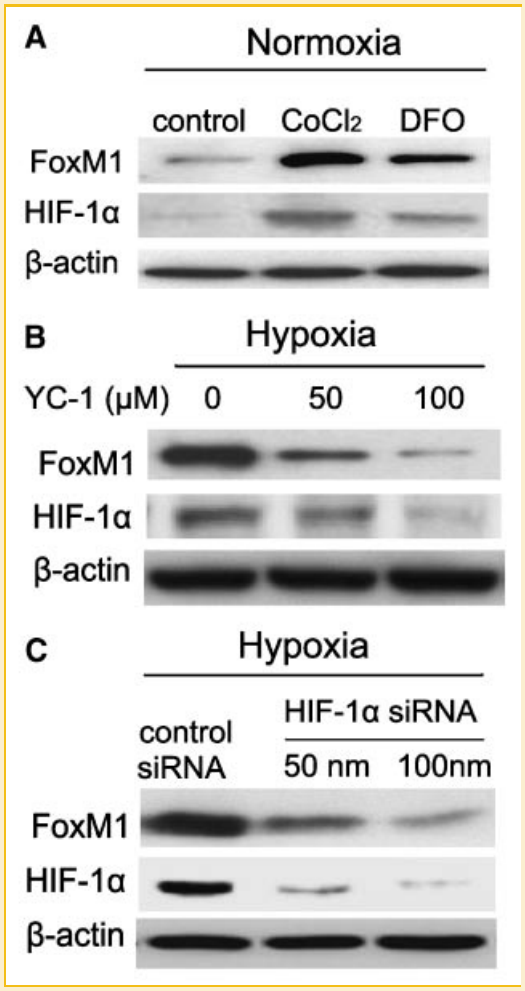

Fig. 2. Association between HIF-1α induction and FoxM1 up-regulation. A: HepG2 cells were treated with deferoxamine (DFO) and CoCl2. HIF-1α and FoxM1 protein levels were analyzed by Western blot. B: Cells were exposed to hypoxia in the presence of increasing concentrations of the guanylyl cyclase inhibitor YC-1 (HIF-1α inhibition), and the protein levels of HIF-1α and FoxM1 were determined. C: HepG2 cells were exposed to hypoxic conditions with or without treatment of control siRNA or siHIF-1α, and the protein levels of FoxM1 and HIF-1α were measured.

were incubated with YC-1 (HIF-1α inhibitor) [Yeo et al., 2003] or siHIF-1α (5′-TACGTTGTGAGTGGTATTATT-3′) [Carroll and Ashcroft, 2006] at 1% O_2, and protein levels of HIF-1α and FoxM1 were analyzed by Western blot. As shown in Figure 2, inhibiting HIF-1α induction during hypoxia using YC-1 and siHIF-1α abolished hypoxia-mediated FoxM1 up-regulation. These observations suggest that HIF-1 is involved in hypoxia-induced FoxM1 up-regulation.

HYPOXIC INDUCTION OF THE FOXM1 PROMOTER IS DEPENDENT ON HIF-1 CONSENSUS BINDING SITES AND HIF-1α ACTIVATION

To dissect the response of FoxM1 promoter to hypoxia, serial deletions of the FoxM1 promoter were cloned in the reporter pGL3-Basic plasmid. The 5′-flanking deletion constructs of the FoxM1 promoter, (−1012/+26)FoxM1, (−600/+26)FoxM1, (−330/+26)FoxM1, (−178/+26)FoxM1, and (−58/+26)FoxM1 were transfected into HepG2 cells and maintained overnight in fresh medium. After incubated under normoxia or hypoxia for 24 h, cells were harvested for dual luciferase assay. Deletion of promoter sequence between −1012 and −330 did not reduce the activity, and deletion of sequence between −330 and −178 led to a dramatic reduction of promoter activity (Fig. 3B). Further deletion to −58 bp completely abolished hypoxia-mediated activation of the FoxM1 promoter reporter. These data suggest that the sequence between −330 and −58 is important for hypoxia-induced FoxM1 expression.

Bioinformatic analysis of the promoter of the human FoxM1 has been performed to search for the DNA sequence 5′-(A/G)CGTG-3′ corresponding to the hypoxia-response element (HRE), which is the HIF-1 binding site [Makino et al., 2007]. We identified four HIF-1 consensus binding sites at −271 bp (HIF1-1, inverted), −225 bp (HIF1-2, inverted), −46 bp (HIF1-3, inverted), and −45 bp (HIF1-4, overlapping HIF1-3) (Fig. 3A). To investigate the role of the four HIF-1 binding sites on FoxM1 activation, these four HIF-1 binding sites (HIF1-1, HIF1-2 and HIF1-3/4) were mutated by multiple rounds of PCR. The mutated constructs of the FoxM1 promoter were transfected into HepG2 cells, and the cells were exposed to hypoxic conditions for 24 h. Luciferase assay showed that mutation of the HIF1-2 binding sites did not significantly affect on hypoxia-induced promoter activity, while mutation of HIF1-1 or HIF1-3/4 significantly decreased hypoxia-induced FoxM1 activation (Fig. 3C). Furthermore, mutation of HIF1-1 and HIF1-3/4 binding sites completely abrogated induction of FoxM1 by hypoxia. These results suggest that the HIF1-1 and HIF1-3/4 binding sites are required for the activation of FoxM1 promoter regulated by hypoxia.

To further confirm the role of HIF-1 in activation of FoxM1 promoter, siHIF-1α was used to knockdown the expression of HIF-1α in hypoxic cells. The report constructs were transfected into HepG2 cells, respectively, and culture in hypoxic conditions with or without siHIF-1α treatments. The luciferase results showed that knockdown of HIF-1α significantly decreased the promoter activity with the HIF-1 consensus binding sites (Fig. 3B,C). These data suggest that the HIF-1 consensus binding site and HIF-1α activation are critical for regulation of FoxM1 promoter in hypoxic conditions.

HIF-1α DIRECTLY BINDS TO THE FOXM1 PROMOTER

HIF-1 binds to the HIF-1 binding sites of hypoxia-inducible genes, which is essential for transactivating their transcription [Wang et al., 1995]. To determine if HIF-1 bound to the HIF-1 consensus binding sites identified in the FoxM1 promoter, HIF-1 binding, and competition ELISA assay were performed. After exposed to normoxic or hypoxic conditions for 24 h, the nuclear extracts from indicated cells were isolated and measured for the HIF-1 DNA binding activity. As shown in Figure 4A, the HIF-1 DNA binding activity was significantly increased in hypoxic cells, compared with the HepG2 cells cultured in normoxia. In addition, increased binding activity was observed with the addition of increasing amounts of nuclear extracts. These data suggest that hypoxia increases the HIF-1 DNA binding activity in hypoxic cells.

We then used the hypoxic nuclear extracts for the competition assay. The nuclear extracts from hypoxic HepG2 cells were incubated with the wild-type or mutated oligonucleotides of

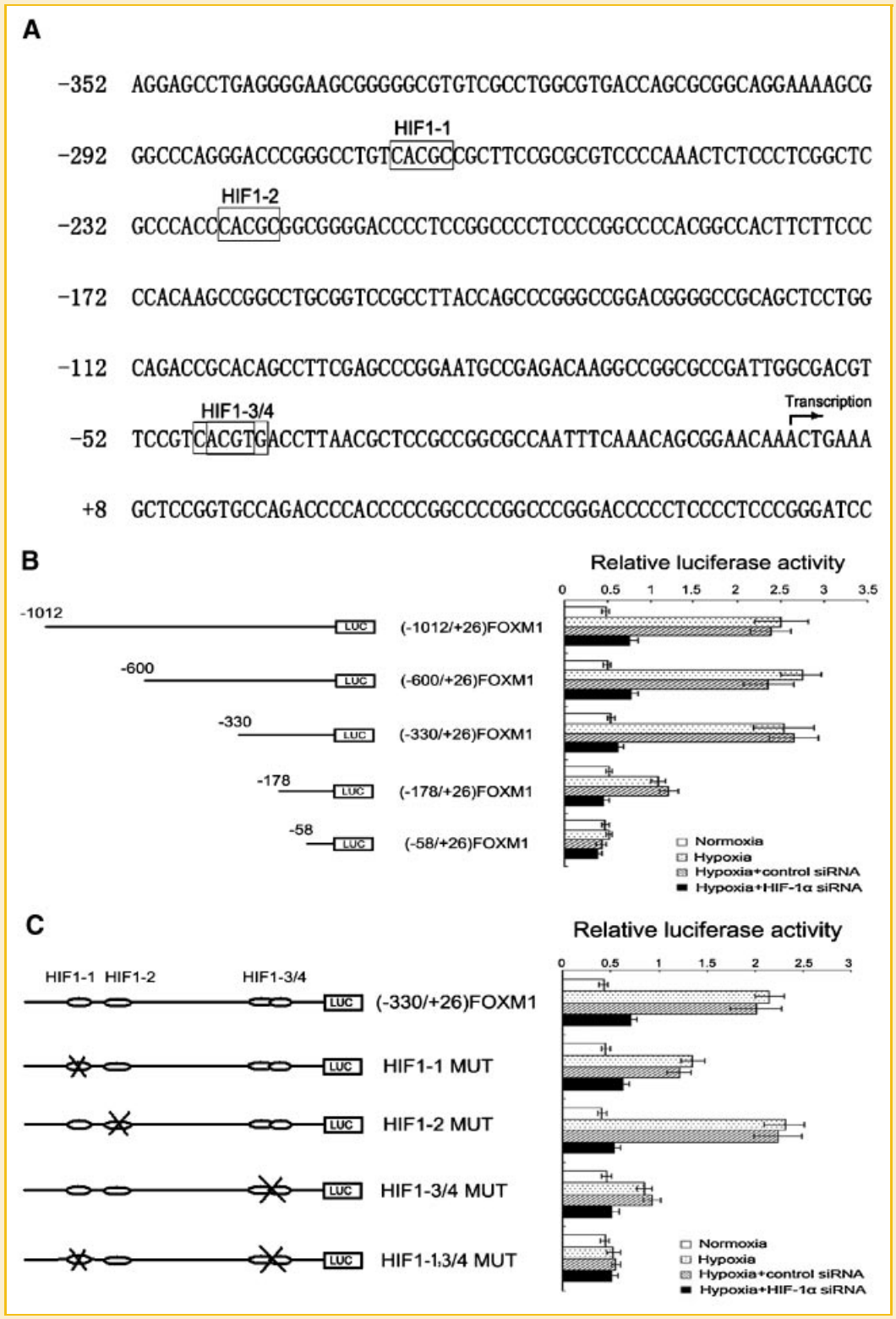

Fig. 3. Involvement of HIF-1 binding site and HIF-1 in hypoxia-induced FoxM1 expression. A: Sequence analysis of the 5′-flanking region of the human FoxM1 gene revealed four putative HIF-1 binding sites. The transcription start site was also indicated in the figure. B: Effects of hypoxia on the activity of 5′ deletion of the FoxM1 promoter. On the left was a schematic representation of the reporter gene constructs assayed by transcient transfection of HepG2 cells, and on the right side the bar graphs represented the relative levels of luciferase activity in each of the transfected samples. C: Effects of hypoxia on the mutations of the FoxM1 promoter. HepG2 cells were transfected with one of the different mutation FoxM1 promoter constructs or wild type FoxM1 promoter. Cells were then incubated under normoxia or hypoxia for 24 h with or without siHIF-1α treatments and luciferase activity was measured. Columns, duplicate determinations in one representative experiment of three; bars, SD.

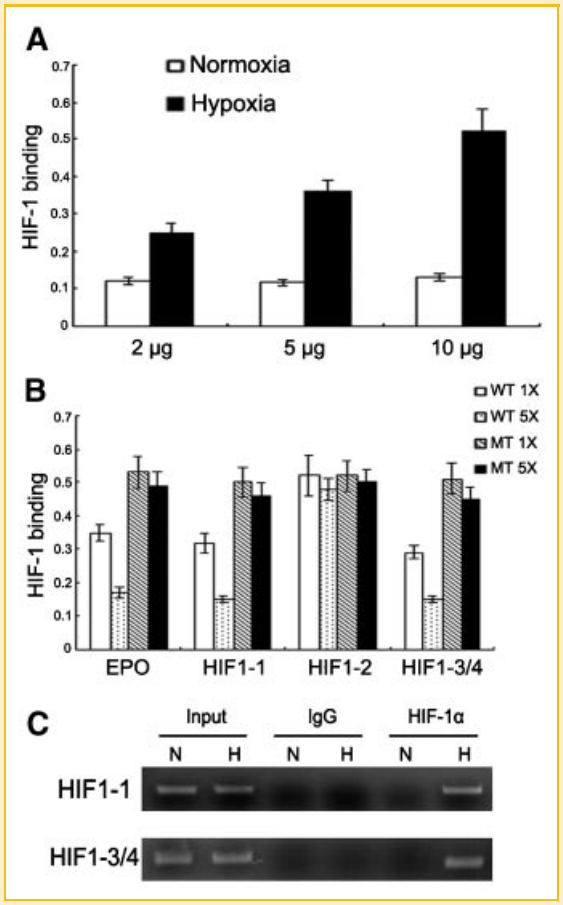

Fig. 4. Direct binding for HIF-1α at the FoxM1 promoter. A: The nuclear extracts (2, 5, and 10 μg) from hypoxic or normoxic cells were added to the wells, and the absorbance was then measured using Microplate Reader Model 680. B: The 20 pmol (1×) or 100 pmol (5×) of wild-type (WT) or the mutant (MT) HIF-1 binding oligonucleotides of 3′ untranlated region of EPO gene or the HIF-1 oligonucleotides of FoxM1 promoter were first added to the appropriate well before addition of 10 μg of the nuclear extracts of hypoxic cells. Columns, duplicate determinations in one representative experiment of three; bars, SD. C: ChIP assay was performed by using an antibody to HIF-1α or a control antibody to pull down the DNA fragment containing the HIF1-1 or HIF1-3/4 binding sites in the FoxM1 promoter from hypoxic (H) or normoxic (N) cells. Input DNA was also amplified to estimate the recovery yields of ChIP.

HIF1-1, HIF1-2, and HIF1-3/4, and then the HIF-1 DNA binding activity were measured. As shown in Figure 4B, pretreatment of nuclear extracts with wild-type oligonucleotides of HIF1-1 or HIF1-3/4 but not HIF1-2 significantly abolished HIF-1α binding to the HIF-1 binding oligonucleotides of EPO. However, the mutant oligonucleotides of FoxM1 promoter did not decrease the HIF-1α binding activity. These results indicate that HIF-1α binds to the HIF1-1 and HIF1-3/4 binding sites derived from the FoxM1 promoter.

Then we performed a ChIP assay to determine whether HIF-1α bound to the FoxM1 promoter in vivo during hypoxia. The DNA fragment containing the HIF1-1 and HIF1-3/4 binding sites in FoxM1 promoter was pulled down from the HepG2 cells exposed to hypoxia by an anti-HIF-1α antibody. However, the FoxM1 promoter PCR fragment containing the HIF1-1 and HIF1-3/4 binding sites was not found in samples pulled down by a control IgG antibody (Fig. 4C). Taken together, these results confirmed that HIF-1α directly binds to the FoxM1 promoter during hypoxia.

FOXM1 IS ESSENTIAL FOR GROWTH AND PROLIFERATION OF HYPOXIC CANCER CELLS

To examine the role of FoxM1 in proliferation of hypoxic cancer cells, we depleted the expression levels of FoxM1 in HepG2, MCF-7, and HeLa cells by transfection with siFoxM1 (5′-GGACCACTTTCCC-TACTTT-3′), which was previously shown to significantly diminish endogenous FoxM1 expression [Kalin et al., 2006]. The siHIF-1α was transfected to the hypoxic cells as a control. Total RNA from these hypoxic cancer cells was prepared at 48 h after transfection with siRNA. As shown in Figure 5A, siFoxM1 and siHIF-1α significantly decreased the mRNA levels of FoxM1, while the control siRNA had little effect on the expression of FoxM1. To investigate the effect of FoxM1 depletion on the growth of hypoxic cancer cells, HepG2, MCF-7, and HeLa cells were transfected with siFoxM1, siHIF-1α, or control siRNA before cultured in hypoxic conditions. The growth rate was determined by MTT assay at 2, 3, or 4 days after transfection. As shown in Figure 5B, all three cancer cell lines exhibited a significant decrease in cell growth after transfection with siFoxM1 or siHIF-1α compared with the cells transfected with control siRNA. These data suggest that depletion of FoxM1 decreased the growth rate of hypoxic cancer cells.

HYPOXIA PROMOTES PROLIFERATION OF TUMOR THROUGH REGULATION GENE EXPRESSION MEDIATED BY FOXM1

To identify the mechanisms of regulation of hypoxic cancer cells proliferation by FoxM1 overexpression, we examined the expression of several cell cycle regulatory genes. It has been demonstrated that FoxM1-deficient hepatocytes diminish S-phase progression through increase in nuclear levels of p21. We exposed HepG2 cells to normoxic or hypoxic conditions, and measured the nuclear levels of p21. Western blot assay results revealed that nuclear levels of p21 significantly decreased in the hypoxic HepG2 cells compared with cells cultured in normoxic conditions. To further investigate the role of FoxM1 in regulation of p21 expression in hypoxic cancer cells, siFoxM1 duplex was used to deplete the expression of FoxM1 in hypoxic HepG2 cells. As shown in Figure 5C, depletion of FoxM1 significantly induced nuclear levels of p21 protein.

Cyclin B1 is a cell cycle regulating protein, which activates cdk1 through complex formation and is critical for progression into mitosis. Cyclin D1 induces the progression of cell cycle from the G1 to S phase by the activation of cdk4 and cdk6 [Holland and Taylor, 2006]. It was also found that the protein levels of cyclin B1 and cyclin D1 increased in the hypoxic cells, and knocking down of FoxM1 by siFoxM1 duplex suppressed the induction of cyclin B1 and cyclin D1 by hypoxia (Fig. 5C). Therefore, these data suggest that FoxM1 regulates the expression of multiple cell cycle genes in hypoxic tumor cells and promotes proliferation of tumor cells.

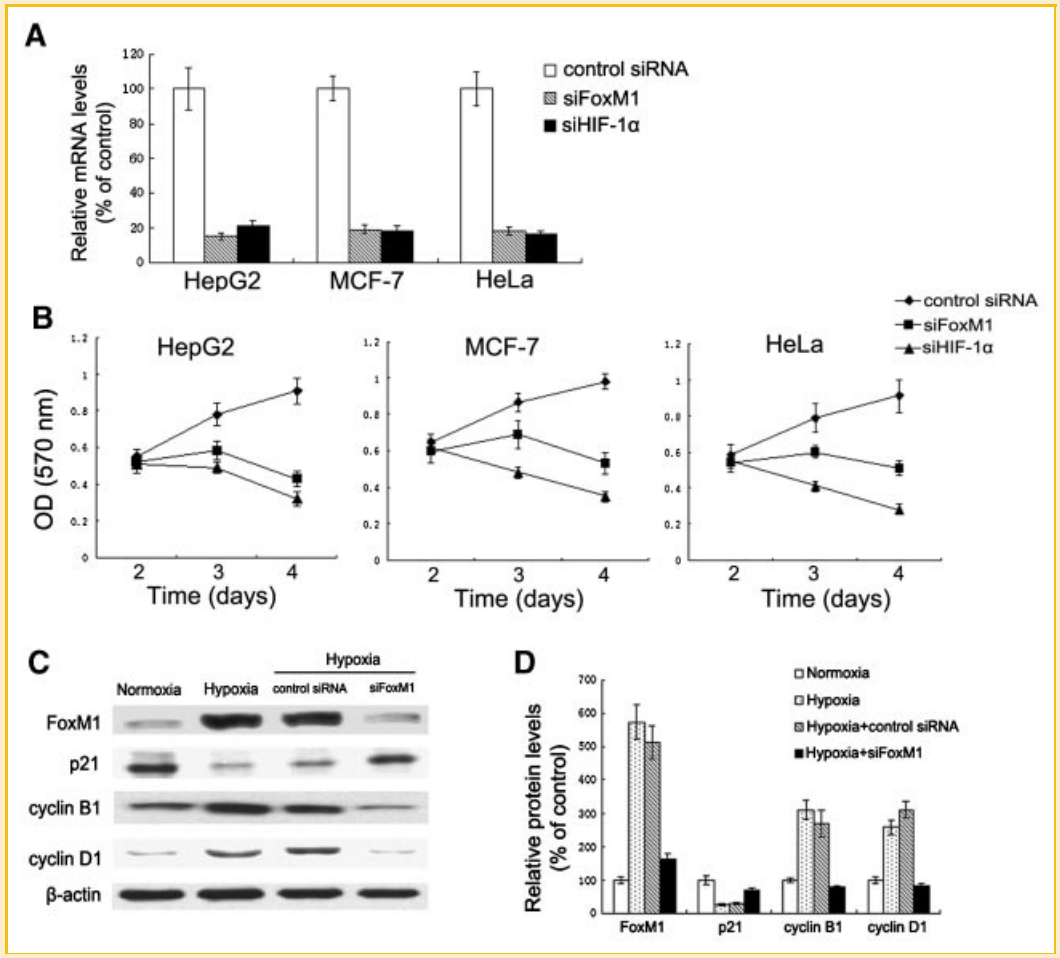

Fig. 5. FoxM1 is essential for growth and proliferation of hypoxic cells. A: HepG2, MCF-7, and HeLa cells were transfected with siFoxM1, siHIF-1α, or control siRNA, and cells were exposed to hypoxia for 48 h. The mRNA levels of FoxM1 and HIF-1α were determined by qRT-PCR. The mRNA levels of HIF-1α and FoxM1 in the cells, which transfected with control siRNA were set to 100%, respectively. The results are the means of three independent experiments. $^{*}P < 0.05$ versus control. B: HepG2, MCF-7, and HeLa cells were transfected with siFoxM1, siHIF-1α, or control siRNA, and then the cells were exposed to hypoxic conditions. At 2, 3, or 4 day after transfection, the growth rate was measured by MTT assay. The results are the means of three independent experiments. $^{*}P < 0.05$ versus control. C,D: HepG2 cells were exposed to hypoxic conditions with or without treatment of control siRNA or siFoxM1 for 48 h, and the protein levels of FoxM1, p21, cyclin B1, and cyclin D1 were measured by Western blot. Densitometric analysis was performed, and the protein levels of FoxM1, p21, cyclin B1, and cyclin D1 in cells cultured in normoxic conditions were set to 100%, respectively.

DISCUSSION

Hypoxia is a common feature of the microenvironment of solid tumors. Adaptation to hypoxia is critical for tumor cell growth and is achieved largely by transcriptional activation of genes that are crucial to tumor progression [Wang et al., 1995]. In the current study, we report for the first time that hypoxia induces FoxM1 expression in tumorigenic cells and show that this up-regulation is mediated by HIF-1. In addition, FoxM1 overexpression in hypoxic conditions promotes proliferation of tumor cells by regulating many FoxM1 target genes expression, which is critical for tumor growth and survival.

Elevated expression of FoxM1 has been found in hepatocellular carcinomas, breast carcinomas, non-small cell lung cancers, and in many other solid tumors. Increased levels of FoxM1 have been seen to accelerate prostate cancer development and progression in mouse models [Kalin et al., 2006]. Furthermore, a large-scale analysis of microarray results revealed that FoxM1 is one of the most common genes overexpressed in a majority of solid tumors [Pilarsky et al., 2004]. Therefore, identifying the regulatory mechanisms that underlie FoxM1 up-regulation is crucial for further understanding of the tumorigenic process and the development of novel approaches for cancer prevention and therapy. Our study showed that hypoxia as a novel tumor microenvironmental factor contributed to FoxM1 up-regulation in cancer cells. HIF-1α, as a key transcription factor induced by hypoxia, was also induced in hypoxic cancer cells, and HIF-1α induction preceded FoxM1 up-regulation. Furthermore, FoxM1 up-regulation was significantly inhibited by HIF-1α depletion. These data indicate that hypoxia up-regulates FoxM1 expression and its up-regulation is mediated by HIF-1.

HIF-1 is a transcription factor that is activated during hypoxia and involved in cell proliferation and apoptosis [Wang et al., 1995]. Indeed, studies have shown that HIF-1 regulates the transcription of several gene clusters that are crucial to tumor progression [Wang et al., 1995]. Our previous studies have shown that hypoxia-induced FoxM1 expression is mediated by HIF-1. To further investigate the molecular mechanisms by which hypoxia induced FoxM1 expression, a series of deletional mutations of FoxM1 promoter assay were performed. It was found that the promoter region between −330 and −58 was critical for the transcriptional response to hypoxia in HepG2 cells. Site-directed mutagenesis confirmed that the HIF1-1 and HIF1-3/4 binding sites, but not HIF1-2 binding site in this region were responsible for FoxM1 transcriptional activation.

HIF-1 binds to the HIF-1 binding sites of hypoxia-inducible genes, which is essential for transactivating their transcription [Wang and Semenza, 1995; Wang et al., 1995]. Sequence-specific binding by HIF-1α to FoxM1 promoter in hypoxic conditions was determined both in vitro and in vivo by HIF-1 binding/competition assay and ChIP assays. Pretreatment with wild-type oligonucleotides of HIF1-1 or HIF1-3/4 significantly abolished HIF-1 binding activity, and the DNA fragment containing the HIF1-1 and HIF1-3/4 binding sites in FoxM1 promoter could be pulled down by anti-HIF-1α antibody. These data indicate that FoxM1 is directly transcriptionally activated in response to hypoxia by HIF-1 binding to the functional HIF-1 binding sites within the proximal FoxM1 promoter.

Recent study has shown that hypoxia increases antiapoptotic potentials in tumor cells, and these effects of hypoxia render tumor cells resistant to various cancer therapies and facilitate the survival of tumor cells [Kim et al., 2004]. The FoxM1 has been shown to be overexpressed in many solid tumors. FoxM1-deficient hepatocytes failed to proliferate and were resistant to the development of hepatic tumors in response to chemical carcinogens [Kalinichenko et al., 2004; Costa et al., 2005]. To investigate the role of FoxM1 up-regulation in proliferation of cancer cells, we used siFoxM1 duplex to knock down expression of FoxM1 in hypoxic cancer cells. It was found that FoxM1 depletion significantly decreased the proliferation rate of cancer cells. These observations suggest that FoxM1 is critical for growth of tumorigenic cells.

In addition, we found that hypoxia diminished nuclear levels of the cdk inhibitor protein p21 and increased the expression of cyclin B1 and cyclin D1, and this effect was mediated by FoxM1 because FoxM1 depletion significantly abolished this effect. p21 is a well-characterized cdk inhibitor that belongs to the Cip/Kip family of cdk inhibitors [Gartel et al., 1996]. It mainly inhibits the activity of cyclin/cdk2 complexes and negatively modulates cell cycle progression [Brugarolas et al., 1999]. In addition, p21 can bind to proliferating cell nuclear antigen thereby blocking DNA synthesis [Waga et al., 1994]. It has been demonstrated that FoxM1 transcriptionally induces Skp2 and Cks1 (specificity subunits of Skp1-Cullin1-F-box ubiquitin ligase complex) leading to the degradation of p21, thereby resulting in cell cycle progression [Wang et al., 2005]. In the present study, we found that hypoxia decreased the p21 level in the nucleus, and depletion of FoxM1 expression significantly abrogated hypoxia-repressed p21 expression. These data indicate that hypoxia probably regulates p21 protein expression, and this regulation is mediated by FoxM1.

Cyclin B1 and cyclin D1 are cell cycle regulating proteins, and facilitate progression through the G2/M and G1/S phage of cell cycle [Ye et al., 1999; Perou et al., 2000]. Several studies have reported that cyclin B1 and cyclin D1 are overexpressed in many cancers, where their overexpression has been associated frequently with poor clinical outcome [Betticher et al., 1996; Egloff et al., 2006]. It has also been demonstrated FoxM1 regulates transcription of cyclin B1 and cyclin D1 [Ye et al., 1999; Wonsey and Follettie, 2005]. In the present study, we found that hypoxia significantly increased cyclin B1 and cyclin D1 expression, and this up-regulation was mediated by induction of FoxM1. Thus, these data suggest that hypoxia regulates growth of tumorigenic cells, which is associated with alterations in FoxM1, p21, cyclin B1, and cyclin D1 expression.

In summary, our findings demonstrate that hypoxia is a stimulus for FoxM1 in tumorigenic cells, and FoxM1 up-regulation involves activation of the promoter of the FoxM1 gene by HIF-1. This induction of FoxM1 leads to promotion of tumor cells proliferation by diminished nuclear levels of p21 protein and increased cyclin B1 and cyclin D1 expression. These studies demonstrate that the FoxM1 transcription factor is induced by hypoxia, and is required for proliferative expansion during tumor progression.

REFERENCES

Betticher DC, Heighway J, Hasleton PS, Altermatt HJ, Ryder WD, Cerny T, Thatcher N. 1996. Prognostic significance of CCND1 (cyclin D1) overexpression in primary resected non-small-cell lung cancer. Br J Cancer 73:294–300.

Brugarolas J, Moberg K, Boyd SD, Taya Y, Jacks T, Lees JA. 1999. Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after γ-irradiation. Proc Natl Acad Sci USA 96:1002–1007.

Carlsson P, Mahlapuu M. 2002. Forkhead transcription factors: Key players in development and metabolism. Dev Biol 250:1–23.

Carroll VA, Ashcroft M. 2006. Role of hypoxia-inducible factor (HIF)-1α versus HIF-2α in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: Implications for targeting the HIF pathway. Cancer Res 66:6264–6270.

Costa RH, Kalinichenko VV, Holterman AX, Wang X. 2003. Transcription factors in liver development, differentiation, and regeneration. Hepatology 38:1331–1347.

Costa RH, Kalinichenko VV, Major ML, Raychaudhuri P. 2005. New and unexpected: Forkhead meets ARF. Curr Opin Genet Dev 15:42–48.

Egloff AM, Vella LA, Finn OJ. 2006. Cyclin B1 and other cyclins as tumor antigens in immunosurveillance and immunotherapy of cancer. Cancer Res 66:6–9.

Gartel AL, Serfas MS, Tyner AL. 1996. p21-negative regulator of the cell cycle. Proc Soc Exp Biol Med 213:138–149.

Giaccia AJ, Simon MC, Johnson R. 2004. The biology of hypoxia: The role of oxygen sensing in development, normal function, and disease. Genes Dev 18:2183–2194.

Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, Gallick GE. 2005 HIF-1α, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene 24:3110–3120.

Harris AL. 2002. Hypoxia—A key regulatory factor in tumour growth. Nat Rev Cancer 2:38–47.

Holland AJ, Taylor SS. 2006. Cyclin-B1-mediated inhibition of excess separase is required for timely chromosome disjunction. J Cell Sci 119:3325–3336.

Huang WJ, Liu ZC, Wei W, Wang GH, Wu JG, Zhu F. 2006. Human endogenous retroviral pol RNA and protein detected and identified in the blood of individuals with schizophrenia. Schizophr Res 83:193–199.

Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. 2002. Hsp90 regulates a VHL-independent HIF-1αdegradative pathway. J Biol Chem 277:29936–29944.

Jiang BH, Zheng JZ, Leung SW, Roe R, Semenza GL. 1997. Transactivation and inhibitory domains of hypoxia inducible factor 1α. Modulation of transcriptional activity by oxygen tension. J Biol Chem 272:19253–19260.

Kalin TV, Wang IC, Ackerson TJ, Major ML, Detrisac CJ, Kalinichenko VV, Lyubimov A, Costa RH. 2006. Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. Cancer Res 66: 1712–1720.

Kalinichenko VV, Major ML, Wang X, Petrovic V, Kuechle J, Yoder HM, Dennewitz MB, Shin B, Datta A, Raychaudhuri P, Costa RH. 2004. Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. Genes Dev 18:830–850.

Kim M, Park SY, Pai HS, Kim TH, Billiar TR, Seol DW. 2004. Hypoxia inhibits tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by blocking Bax translocation. Cancer Res 64:4078–4081.

Kim IM, Ackerson T, Ramakrishna S, Tretiakova M, Wang IC, Kalin TV, Major ML, Gusarova GA, Yoder HM, Costa RH, Kalinichenko VV. 2006. The forkhead box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. Cancer Res 66:2153–2161.

Laoukili J, Kooistra MR, Bras A, Petrovic V, Major ML, Park HJ, Tan Y, Ackerson T, Costa RH. 2005. FoxM1 is required for execution of the mitotic programme and chromosome stability. Nat Cell Biol 7:126–136.

Liu M, Dai B, Kang SH, Ban K, Huang FJ, Lang FF, Aldape KD, Xie TX, Pelloski CE, Xie K, Sawaya R, Huang S. 2006. FoxM1B is overexpressed in human glioblastomas and critically regulates the tumorigenicity of glioma cells. Cancer Res 66:3593–3602.

Mahon PC, Hirota K, Semenza GL. 2001. FIH-1: A novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15:2675–2686.

Makino Y, Uenishi R, Okamoto K, Isoe T, Hosono O, Tanaka H, Kanopka A, Poellinger L, Haneda M, Morimoto C. 2007. Transcriptional up-regulation of inhibitory PAS domain protein gene expression by hypoxia-inducible factor 1 (HIF-1): A negative feedback regulatory circuit in HIF-1-mediated signaling in hypoxic cells. J Biol Chem 282:14073–14082.

Marsden I, Jin C, Liao X. 1998. Structural changes in the region directly adjacent to the DNA binding helix highlight a possible mechanism to explain the observed changes in the sequence-specific binding of winged helix proteins. J Mol Biol 278:293–299.

Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y. 2001. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: Identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res 61:2129–2137.

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Aksien LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. 2000. Molecular portraits of human breast tumors. Nature 406:747–752.

Pilarsky C, Wenzig M, Specht T, Saeger HD, Grutzmann R. 2004. Identification and validation of commonly overexpressed genes in solid tumors by comparison of microarray data. Neoplasia 6:744–750.

Rivard A, Berthou-Soulie L, Principe N, Kearney M, Curry C, Branellec D, Semenza GL, Isner JM. 2000. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 275:29643–29647.

Semenza GL. 2000. HIF-1: Mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88:1474–1480.

Waga S, Hannon GJ, Beach D, Stillman B. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369:574–578.

Wang GL, Semenza GL. 1995. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270:1230–1237.

Wang GL, Jiang BH, Rue EA, Semenza GL. 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.

Wang X, Kiyokawa H, Dennewitz MB, Costa RH. 2002a. The forkhead box m1b transcription factor is essential for hepatocyte DNA replication and mitosis during mouse liver regeneration. Proc Natl Acad Sci USA 99:16881–16886.

Wang X, Krupczak-Hollis K, Tan Y, Dennewitz MB, Adami GR, Costa RH. 2002b. Increased hepatic forkhead box M1B (FoxM1B) levels in old-aged mice stimulated liver regeneration through diminished p27Kip1 protein levels and increased Cdc25B expression. J Biol Chem 277:44310–44316.

Wang IC, Chen YJ, Hughes D, Petrovic V, Major ML, Park HJ, Tan Y, Ackerson T, Costa RH. 2005. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-1) ubiquitin ligase. Mol Cell Biol 25:10875–10894.

Wonsey DR, Follettie MT. 2005. Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. Cancer Res 65:5181–5189.

Yao KM, Sha M, Lu Z, Wong GG. 1997. Molecular analysis of a novel winged helix protein, WIN. Expression pattern, DNA binding property, and alternative splicing within the DNA binding domain. J Biol Chem 272:19827–19836.

Ye H, Holterman A, Yoo KW, Franks RR, Costa RH. 1999. Premature expression of the winged helix transcription factor HFH-11B in regenerating mouse liver accelerates hepatocyte entry into S-phase. Mol Cell Biol 19:8570–8580.

Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS, Park JW. 2003. YC-1: A potential anticancer drug targeting hypoxia-inducible factor 1. J Natl Cancer Inst 95:516–525.