HIF-1 (hypoxia inducible factor 1) performs a crucial role in mediating the response to hypoxia. However, other transcription factors are also capable of regulating hypoxia-induced target-gene transcription. In a previous report, we demonstrated that the transcription factor ATF-2 (activating transcription factor 2) regulates hypoxia-induced gene transcription, along with HIF-1α. In the present study, we show that the protein stability of ATF-2 is induced by hypoxia and the hypoxia-mimic CoCl₂ (cobalt chloride), and that ATF-2 induction enhances HIF-1α protein stability via direct protein interaction. The knockdown of ATF-2 using small interfering RNA and translation-inhibition experiments demonstrated that ATF-2 plays a key role in the maintenance of the expression level and transcriptional activity of HIF-1α. Furthermore, we determined that ATF-2 interacts directly with HIF-1α both in vivo and in vitro and competes with the tumour suppressor protein p53 for HIF-1α binding. Collectively, these results show that protein stabilization of ATF-2 under hypoxic conditions is required for the induction of the protein stability and transcriptional activity of HIF-1α for efficient hypoxia-associated gene expression.

Key words: activating transcription factor 2 (ATF-2), hypoxia inducible factor-1 (HIF-1), hypoxia, protein stability.
be activated under hypoxic stress conditions [28], but its relationship with HIF-1α has yet to be elucidated.

In the present study, we demonstrate that ATF-2 is a crucial component of the HIF-1α regulatory machinery by virtue of its association with HIF-1α regulatory hypoxic conditions. ATF-2 protein levels were induced under hypoxic conditions via the up-regulation of ATF-2 protein stability along with its phosphorylation. Furthermore, using RNAi (RNA interference) methods, we determined that the induction of ATF-2 occurs in an HIF-1α-independent manner, but the abundantly expressed ATF-2 is important for HIF-1α expression and its transcriptional activity. ATF-2 overexpression in the presence of CoCl2 (cobalt chloride) is sufficient to augment HIF-1α protein stability. We also noted that ATF-2 can bind directly to HIF-1α, thereby competing with p53, and this interaction appeared to be essential for the full induction of HIF-1α under hypoxic circumstances.

**MATERIALS AND METHODS**

**Cell culture and hypoxic conditions**

Human hepatocarcinoma HepG2 cells were cultured in minimal essential medium (Gibco BRL) with 10% (v/v) heat-inactivated FBS (fetal bovine serum; Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO2 and were re-fed every 2 days. Chang liver cells and HuH7 hepatocarcinoma cells were grown in DMEM (Dulbecco’s modified Eagle’s medium; Gibco BRL), supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin/streptomycin. To achieve a hypoxic atmosphere, cells were placed in a hypoxic chamber supplying 2% O2 and 5% CO2 at 37°C for the times indicated in the Results section. To mimic hypoxia, cells were incubated in the presence of 200 μM CoCl2 (Sigma–Aldrich). When cells were subjected to hypoxia or CoCl2, all of the cell medium was replaced with serum-free medium.

**Transfection and analysis of luciferase activity**

For transfectional transactivation experiments, Chang liver cells were transfected at 30–50% confluence using PolyFect transfection reagent (Qiagen). Briefly, the cells were seeded in 24-well plates at a density of 4 × 105 cells/well. Cells were grown overnight at 37°C and then transfected in DMEM containing 2 μl of PolyFect reagent, 200 ng of reporter plasmid and 50–100 ng of HIF-1α. ATF-2 expression vectors or empty control plasmid to a total DNA amount of 400 ng. Transfection efficiency was normalized by co-transfection of 100 ng of HIF-1α, HIF-1α/p53 expression vector. After incubation with DNA precipitates for 3 min and analysed by SDS/PAGE (8–10% gel) transferred on to PVDF membranes. Upon completion of transfer, the membranes were probed with anti-pHIF-1α (Thr23) (1:1000; Cell Signaling Technologies), anti-HIF-1α (1:500; BD Bioscience), anti-VEGF (1:500; BD Bioscience), anti-cyclin D1 (1:5000; Pharmingen), anti-p27 (1:3000; Santa Cruz Biotechnology), anti-NF-κB (nuclear factor κB; 1:3000; Santa Cruz Biotechnology) and anti-p53 (1:50000; Oncogene) antibodies. Bands were detected using Advanced ECL® Western Blotting Detection Reagents (Amersham Biosciences). Loading was checked with anti-β-actin antibody (1:20000; Santa Cruz Biotechnology).

**RT–PCR (reverse transcription–PCR) analysis**

Total RNA from cells was isolated using TRIzol® reagent (Life Technologies) according to the manufacturer’s instructions. First-strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase with 5 μg of each DNA-free total RNA sample and oligo(dT)15 (Life Technologies) according to the manufacturer’s instructions. Equal amounts of cDNA were subsequently amplified by PCR in a 50 μl reaction volume containing 1× PCR buffer, 200 μM dNTPs, 10 μM specific primer for HIF-1α (sense 5′-ACCAACCTCAGTGGGGAT-3′, antisense 5′-GACTGAGGAAATCTTGCT-3′), VEGF (sense 5′-CTTGCCCTTGCTGCTTAC-3′, antisense 5′-ATTCAGTTGGGTAGAC-3′), β-actin (sense 5′-GACTACCTCATGAAGATC-3′, antisense 5′-GAATTTCTGCGAGGAAATAC-3′), 1.25 units of Taq DNA polymerase (PerkinElmer). Amplification products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining under UV transillumination.

**Immunoprecipitation**

Nuclear extracts (250 μg of protein) from HepG2 cells (transfected or non-transfected) were incubated with 1 μg of anti-ATF-2 antibody or anti-HIF-1α antibody as indicated at 4°C for 2 h with gentle agitation. Immune complexes were collected on Protein G–Sepharose beads (Gibco BRL). After washing three times with 0.7 ml of protein lysis buffer [1% (v/v) Triton X-100 and 1% (w/v) deoxycholate in PBS], the precipitates were boiled with an equal volume of 2× LSB (Laemmli sample buffer) at 100°C for 3 min and analysed by SDS/PAGE (8–10% gel), followed by Western blotting using anti-HIF-1α antibody.

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GST (glutathione transferase) pull-down assay

The GST-fused ATF-2 deletion constructs or GST alone were expressed in *Escherichia coli* DH5-α cells, bound to glutathione-Sepharose-4B beads (Amersham Biosciences) and incubated with haemagglutinin-tagged HIF-1α proteins expressed by *in vitro* translation using the TNT® T7 Quick-Coupled Transcription-Translation System (Promega), with conditions as described by the manufacturer. Specifically bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0 and analysed by SDS/PAGE (8–10% gel) and Western blotting.

In vivo ubiquitination assay

HepG2 cells in 60-mm-diameter plates (2 × 10⁵ cells/plate) were transfected with His₆-ubiquitin. (2 μg) or with other expression plasmids using Lipofectamine™ reagent (Invitrogen). After 42 h of transfection, cells were washed and the medium was replaced with fresh medium containing 20 μM MG132 (a proteasome inhibitor) and incubated in normoxic or hypoxic conditions in the presence or absence of CoCl₂ or DP (2,2′-dipyridyl) for 4 h. Cells from each plate were harvested and split into two aliquots, one for standard immunoblot analysis and the other for detection of ubiquitinated proteins using Ni-NTA (Ni²⁺-nitrilotriacetate) beads (Qiagen). Cell pellets were lysed in buffer A (6 M guanidinium chloride, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris/HCl, pH 8.0, 5 mM imidazole and 10 mM 2-mercaptoethanol) and incubated with Ni-NTA beads at room temperature (27°C) for 4 h. Beads were washed once with buffer A, once with buffer B (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris/HCl, pH 8.0, and 10 mM 2-mercaptoethanol) and once with buffer C (8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris/HCl, pH 6.3, and 10 mM 2-mercaptoethanol). Proteins were eluted from beads with buffer D (200 mM imidazole, 0.15 M Tris/HCl, pH 6.7, 30% glycerol, 0.72 M 2-mercaptoethanol and 5% SDS). The eluted proteins were analysed by immunoblotting for the polyubiquitination of ATF-2 with polyclonal anti-ATF-2 antibody (1:5000), and for the polyubiquitination of HIF-1α with monoclonal anti-HA antibody (1:10000) (Cell Signaling Technology).

RESULTS

**Hypoxia and CoCl₂ induce ATF-2 expression along with its phosphorylation**

In order to elucidate the effects of hypoxic stress conditions on the expression of ATF-2 protein, HepG2 human hepatocarcinoma cells were incubated under hypoxic conditions (2% O₂). Hypoxic incubation induced an increase in the level of HIF-1α protein as had been expected and, interestingly, cells under hypoxic conditions showed an induction of ATF-2 protein, along with its phosphorylation (Figure 1A). As the application of CoCl₂ treatment to cells had a similar effect on ATF-2 expression to hypoxic conditions, we demonstrated further that this induction of ATF-2 by hypoxic stress generally occurs in other types of hepatic cells (Figure 1C).

**CoCl₂ increases the level of ATF-2 protein via the induction of protein stability**

In an attempt to acquire a better understanding of the processes inherent to ATF-2 accumulation under hypoxic conditions, we assessed the effects of CoCl₂ treatment on the induction of ATF-2 mRNA. HepG2 cells were treated with CoCl₂ for 4 h and utilized for RT–PCR and immunoblot assays. Whereas ATF-2 mRNA was detected at similar levels in CoCl₂-treated cells compared with controls, the ATF-2 protein was up-regulated in the presence of CoCl₂ (Figure 2A). These results suggested that the induction of ATF-2 by CoCl₂ does not occur at the level of transcription or RNA stability.

In order to determine whether hypoxic conditions can affect ATF-2 protein stability, we attempted to ascertain whether or not the ATF-2 protein induced by CoCl₂ is stable under normal conditions. HepG2 cells were treated with CoCl₂ for 4 h and then the medium was exchanged with fresh medium to mimic reoxygenation after hypoxic stress. CoCl₂ induced higher levels of HIF-1 and ATF-2 proteins, but the removal of CoCl₂ from cells resulted in an immediate decline in ATF-2 protein levels along with HIF-1α (Figure 2B), thereby suggesting that the ATF-2 protein induced by CoCl₂ is unstable in the absence of CoCl₂. We further verified the effect of CoCl₂ on ATF-2 protein stability using the translation inhibitor cycloheximide. HepG2 cells were incubated in the presence of CoCl₂ and the cells were subsequently exposed to cycloheximide in the presence or absence of CoCl₂. As shown in Figure 2(C), when the CoCl₂ was removed, the ATF-2 levels induced by CoCl₂ declined gradually in a time-dependent manner. On the other hand, in the presence of CoCl₂, the cells maintained...
Hypoxia induces ATF-2 protein stability, but not its transcription

(A) HepG2 cells were incubated in the presence or absence of CoCl$_2$, and ATF-2 mRNA levels were analysed using RT–PCR. As indicated, ATF-2 mRNA levels were partially decreased in the presence of CoCl$_2$ (left panel), whereas the immunoblot control for the RT–PCR demonstrated a strong induction of the ATF-2 protein (right panel). The results from RT–PCR were quantified and normalized relative to the β-actin mRNA levels using ImageJ version 1.40 (National Institutes of Health Image). The results shown are from one experiment representative of four independent experiments. The values are expressed as the means ± S.D. (n = 4), P < 0.00005 compared with the normoxic cells. (B) HepG2 cells were incubated for 4 h in either normoxic (untreated) or hypoxic medium (CoCl$_2$-treated), after which the hypoxic medium of the cells were replaced with normal fresh medium, and the cells were incubated for the indicated times. The cells were then analysed by immunoblotting for ATF-2 and HIF-1α protein levels. (C) HepG2 cells were incubated in either the presence or absence of CoCl$_2$, after which CoCl$_2$-pretreated cells were washed and exposed to the protein-translation inhibitor cycloheximide (CHX; 50 μg/ml) with or without CoCl$_2$, then incubated for the indicated times. The cells were then analysed by immunoblotting for ATF-2 and HIF-1α protein levels. (D) HepG2 cells were transfected with plasmids encoding His$_6$–ubiquitin (pHis-Ub). After transfection, cells were washed, the medium was replaced with fresh medium containing 20 μM MG132, and the cells subjected to 200 μM CoCl$_2$, 100 μM DP and hypoxia (2 % O$_2$) as indicated. After 4 h of incubation, cells were harvested and used for in vivo ubiquitination assay as described in Materials and methods section. Ubiquitinated ATF-2 was detected via Western blot analysis with ATF-2 antibody (right panel). Expression levels of HIF-1α, ATF-2, His–ubiquitin and β-actin in input total extracts were visualized via Western blot analysis (left panel).
their increased ATF-2 levels after 1 h of incubation. In order to determine further whether the maintenance of ATF-2 levels in the presence of CoCl₂ is attributable to a blockade of protein degradation, the cells were treated with 20 μM MG132 in the presence or absence of CoCl₂ (Figure 2D). As expected, CoCl₂ alone was sufficient to induce ATF-2 in normal cells. The levels of ATF-2 protein were the same in both the CoCl₂-treated cells and the untreated cells in the presence of MG132. Finally, we performed an in vivo ubiquitination assay to elucidate whether the hypoxic condition affects ubiquitination of ATF-2 (Figure 2E). Under hypoxia, ubiquitination of ATF-2 was decreased and this phenomenon was reproduced by treatment of cells with CoCl₂ and DP (an iron chelator and HIF-PHD inhibitor similar to CoCl₂). These results demonstrated that the induction of ATF-2 protein under hypoxic conditions results in the inhibition of ubiquitination-associated proteolysis.

Induction of ATF-2 by CoCl₂ is HIF-1-independent but is important for HIF-1 regulation under hypoxic conditions

In order to identify the functional interaction of protein stabilization between ATF-2 and HIF-1α, we conducted a gene-silencing assay using siRNA targeting ATF-2 and HIF-1α. As shown in Figure 3(A), HepG2 cells transiently transfected with the HIF-1α siRNA showed a marked loss of HIF-1α, whereas the same siRNA exerted no ATF-2 induction effects in response to CoCl₂ treatment. This result implies that the induction of ATF-2 by CoCl₂ is an HIF-1α-independent event. We explored further the effects of siRNA targeting of ATF-2 in order to elucidate the function of induced ATF-2 on HIF-1α expression by CoCl₂. HepG2 cells transfected with siRNA targeting ATF-2 reduced the level of the ATF-2 protein by approx. 60% and, interestingly, the level of HIF-1α induced by CoCl₂ was decreased dramatically.

In an effort to characterize further the change in the transcriptional activity of HIF-1α by ATF-2 silencing, we conducted luciferase assays using a hypoxia-sensitive reporter-gene vector encoding the luciferase gene under the control of a minimal promoter harbouring three copies of the HRE (hypoxia-response element) from the erythropoietin gene. HepG2 cells were transfected with control siRNA and a plasmid for ATF-2 and HIF-1α production. The luciferase activity increased by approx. 1.7- and 2.7-fold respectively, but when they were introduced together, promoter activity was increased in a synergistic manner, by up to 80-fold. When the cells were treated with CoCl₂, the reporter activity was increased by 5.7-fold, thereby indicating that ATF-2 overexpression can partially mimic the hypoxic conditions induced by CoCl₂. The treatment of cells with CoCl₂ synergistically increased reporter gene activity above that of the untreated cells.

In an effort to further verify whether ATF-2 overexpression increases HIF-1α targeted gene expression, Chang liver cells were transfected with VEGF-promoter reporter constructs and a transactivation assay was conducted. The overexpression of ATF-2 and HIF-1α alone were shown to increase VEGF promoter activity by approx. 1.7- and 2.7-fold respectively, but when they were introduced together, promoter activity was increased in a synergistic manner, by up to 30-fold (Figure 5B). The effect of ATF-2 on HIF-1α-mediated gene expression was confirmed further using RT–PCR methods. The vector encoding ATF-2 and HIF-1α were transiently transfected into HepG2 cells in the presence or absence of CoCl₂ treatment (Figure 5C). As a result, ATF-2 overexpression alone was not sufficient to induce VEGF gene expression, but when ATF-2 was added along with HIF-1α, HIF-1α-mediated VEGF gene expression increased.

Interaction of ATF-2 with HIF-1α increases the protein stability and transcriptional activity of HIF-1α

In an effort to assess whether the HIF-1α regulation mediated by ATF-2 requires physical interaction in the cells, HepG2 cells were treated with CoCl₂ or were incubated under hypoxic conditions for 4 h, then subjected to immunoprecipitation (Figure 6A). As we had expected, hypoxic stress appears to be sufficient for the formation of the ATF-2–HIF-1α complex. The transactivation domain of ATF-2 is located at region 1–323 and harbours domains for phosphorylation by SAPK (stress-activated protein kinase), as well as the HAT (histone acetyl transferase)-activity domain, as documented earlier. The remaining portion of ATF-2 (region 323–492) harbours the bZIP domain, which is crucial for dimerization with an AP-1 partner (c-Jun, for instance) and

ATF-2 induces HIF-1α-dependent transactivation by CoCl₂

In order to determine whether the increased HIF-1α stability mediated by ATF-2 induction is directly associated with the expression of the HIF-1 target genes, we conducted luciferase assays using an HRE-promoter reporter plasmid with expression vectors for ATF-2 and HIF-1α (Figure 5A). Under normal conditions, HIF-1α overexpression enhanced luciferase activity by approx. 12-fold as we had anticipated and, interestingly, the overexpression of ATF-2 itself also increased luciferase activity by approx. 3-fold. When ATF-2 and HIF-1α were transfected together, luciferase activity was synergistically increased, by up to 80-fold. When the cells were treated with CoCl₂, the reporter activity was increased by 5.7-fold, thereby indicating that ATF-2 overexpression can partially mimic the hypoxic conditions induced by CoCl₂. The treatment of cells with CoCl₂ synergistically increased reporter gene activity above that of the untreated cells.

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ATF-2 increases HIF-1α protein stability

We then attempted to determine whether ATF-2 directly modulates HIF-1α protein stability. First, we assessed whether ATF-2 overexpression modulates the level of exogenous HA-tagged HIF-1α, via a transient-transfection experiment. HepG2 cells were co-transfected with pCMV–HA–HIF-1α and pCMX–GAL4 or pCMX–GAL4–ATF-2 as indicated, and the cells were incubated in the presence or absence of CoCl₂. As had been expected, overexpressed ATF-2 increased the levels of HIF-1α in the normal and CoCl₂ treatment groups (Figure 4A). The effect of ATF-2 overexpression on HIF-1α protein stability was confirmed further by treatment with cycloheximide (Figure 4B). Western blot analysis to determine the levels of HIF-1α protein demonstrated that the protein stability of HIF-1α was prolonged when the ATF-2 expression vector was transfected, whereas HIF-1α was more sensitive to protein degradation in empty-plasmid-transfected cells. The protein stability of other proteins (cyclin D1, p27 and NF-κB) was studied further to elucidate that increased HIF-1α protein stability mediated by ATF-2 is a specific event. These results demonstrate that ATF-2 up-regulates the expression of HIF-1α via an induced increase in ATF-1α protein stability.

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Figure 3  The induction of ATF-2 is HIF-1-independent, but is crucial for the regulation of HIF-1 under hypoxic conditions

(A) HepG2 cells were incubated for approx. 24 h until they were 50% confluent. The cells were then treated with siRNA to block HIF-1α and ATF-2 expression. The cells were incubated for 40 h under normoxic conditions, after which they were placed in normoxic or CoCl2-containing hypoxic medium for 4 h. Normoxic and CoCl2-treated cells were then analysed by immunoblotting for the indicated proteins. Abbreviations: sc, scrambled siRNA (non-specific siRNA); siH, siRNA against HIF-1α; siA, siRNA against ATF-2. The results shown are representative of three independent experiments. (B) HepG2 cells were transiently transfected with 200 ng of HRE–luciferase reporter plasmid along with 50 ng of pCMVβ–HA–HIF-1α as indicated. After 4 h of transfection, the cells were washed and then treated with non-specific siRNA (open bars) or siRNA against ATF-2 (solid bars). After siRNA treatment, the cells were washed and placed in normoxic or CoCl2-containing hypoxic medium, then analysed for luciferase activity. (C) Scrambled siRNA or siRNA against ATF-2 was applied to the HepG2 cells and after 24 h, the cells were washed and placed in normoxic or hypoxic medium as indicated. After 16 h of incubation, the cells were utilized for RT–PCR analysis. The RT–PCR results were quantified and normalized relative to the β-actin mRNA levels using ImageJ version 1.40 (National Institutes of Health Image). The results shown are from one experiment representative of four independent experiments. The values are expressed as the mean ± S.D. (n = 4); P < 0.03 compared with the scrambled siRNA transfectants exposed to CoCl2. Abbreviations: CA9, carbonic anhydrase 9; PAI-1, plasminogen activator inhibitor 1. (D) HepG2 cells were used for the same set of experiments (A–C) using hypoxia (2% O2) instead of CoCl2.
ATF-2 increases protein stability of HIF-1α

Figure 4 ATF-2 induces HIF-1α protein stability

(A) HepG2 cells were transiently transfected with 1 μg of pCMVβ–HA–HIF-1α coupled with 2 μg of pCMX–GAL4–ATF-2 or pCMX–GAL4 empty vector as indicated. After 40 h of transfection, the cells were placed in normoxic or 200 μM CoCl2-containing hypoxic medium for 4 h. The total protein extracts were acquired and subjected to immunoblotting (30 μg per lane) with anti-HIF-1α, anti-HA, anti-ATF-2 and anti-GAL4 antibodies as indicated. An antibody against β-actin was employed as a loading control. (B) HepG2 cells were transiently transfected with 2 μg of pCMX-I empty vectors or pCMX–ATF-2 as indicated. After 40 h of transfection, the cells were washed and placed in normoxic or 200 μM CoCl2-containing hypoxic medium as indicated, then incubated for an additional 4 h. At 1 h prior to cell harvest, the cells were treated with DMSO or 70 μg/ml of cycloheximide and incubated for the times indicated. Total cell extracts were utilized for immunoblotting with anti-HIF-1α, anti-p27, anti-cyclin D1, anti-NF-κB and anti-ATF-2 antibodies.

...for DNA binding, and the phosphorylation site mediated by ATM (ataxia telangiectasia mutated)/ATR (ataxia telangiectasia mutated- and Rad3-related) in response to genotoxic radiation. In order to determine whether or not ATF-2 binds directly to HIF-1α and to delineate the region of ATF-2 that interacts with HIF-1α, we prepared bacterially extracted GST–ATF-2 full, N-terminal (1–323 amino acids) and C-terminal (323–492 amino acids) proteins, and conducted a GST pull-down assay along with in vitro translated HIF-1α. As shown in Figure 6(B), GST–ATF-2 full and N-terminal-domain proteins interacted with HIF-1α, but not with the C-terminal protein and GST. These results imply that under hypoxic conditions, ATF-2 and HIF-1α can form a functional and physical complex, and that the direct interaction is mediated via the N-terminal domain of ATF-2. Both immunoprecipitation and in vitro GST pull-down assay results show that HIF-1α may co-localize with ATF-2.

In an effort to further elucidate whether the DNA-binding activity of ATF-2 is essential for the regulation of HIF-1α target-gene expression, we conducted a luciferase assay using these two ATF-2 deletion mutants. ATF-2(N), which harbours the N-terminus of ATF-2, exerts similar effects on luciferase activity as the wild-type protein, but ATF-2(C) did not increase HIF-1α-dependent transactivation (Figure 6C). These results show that the ATF-2-mediated activation of HIF-1α occurs independently of ATF-2 DNA-binding activity. Collectively, these results show that the N-terminal region of ATF-2 binds directly to HIF-1α, thus ATF-2 performs a crucial function as a regulator that enhances the protein stability and transcriptional activity of HIF-1α.

ATF-2 blocks p53-mediated HIF-1α degradation through inhibiting HIF-1α interaction with p53

In order to elucidate the manner in which ATF-2 regulates HIF-1α protein stability, we assessed the effects of ATF-2 with other types of HIF-1α regulators. The tumour suppressor protein p53 is induced under hypoxic conditions and has been shown to function as a negative regulator of HIF-1α [6]. p53 can interact directly with HIF-1α, resulting in the Mdm2-mediated ubiquitination of HIF-1α. Thus we attempted to determine whether or not ATF-2 could influence the interaction between HIF-1α and p53. Under hypoxia, ectopic ATF-2 expression in the transfected HepG2 cells...
abolished the interaction between p53 and HIF-1α, as revealed by immunoprecipitation with the HIF-1α antibody (Figure 7A).

In order to confirm further that this inhibitory effect of ATF-2 on the interaction between HIF-1α and p53 is linked directly to HIF-1α protein stability, we attempted to determine whether ATF-2 inhibits p53-mediated HIF-1α degradation. Ectopic p53 expression was sufficient to reduce the levels of exogenous HA-tagged HIF-1α protein, as we had expected, and ATF-2 expression was also reduced by p53. In cells co-transfected with p53- and ATF-2-expressing vectors, HIF-1α protein levels were recovered in an ATF-2-expression-dependent manner (Figure 7B).

We ascertained further that this competitive effect of ATF-2 with p53 on HIF-1α interaction is associated with HIF-1α-mediated transcription (Figure 7C).

In order to determine whether the competition of ATF-2 with p53 for HIF-1α binding can be linked to ubiquitination of HIF-1α, we tried to examine ubiquitination through an in vivo assay (Figure 7D). The ectopic expression of p53 increased polyubiquitination of HIF-1α under hypoxia, but co-transfection of ATF-2 with p53 suppressed ubiquitination in a dose-dependent manner. These results show that ATF-2 increases HIF-1α protein stability by inhibiting p53-mediated HIF-1α degradation and that interaction of ATF-2 with HIF-1α is associated with this inhibition.

We also performed a reporter assay using sh (small-hairpin) RNA targeting p53 to elucidate the effect of ATF-2 overexpression under p53-depleted conditions (Figure 8). First, we confirmed that ectopic expression of sh-p53 decreased the level of p53 protein in a dose-dependent manner and that 3 μg of sh-p53 was enough to eliminate almost all p53 protein from Chang liver cells (Figure 8, upper panel). In the HRE-reporter experiments, for which cells were transfected with sh-EGFP (enhanced green fluorescent protein) plasmid as a negative control, ectopic expression of ATF-2 induced transcriptional activity of HIF-1α from 15-fold to 40-fold under normoxia, and from 70-fold to 132-fold under hypoxia (Figure 8, lower panel). On the other hand, ectopic expression of sh-p53 itself can induce basal activity of HRE-reporter constructs and, in the absence of p53, increased ATF-2 had no effect on HIF-1α-mediated transcription, indicating that inhibition of the p53–HIF1α interaction mediated by increased ATF-2 under hypoxia is crucial.

**DISCUSSION**

The principal objective of the present study was to assess thoroughly the involvement of ATF-2 in HIF-1α-mediated gene regulation under hypoxic conditions. This report is a continuation of our previous work, in which we demonstrated the formation of the functional complex of ATF-2–HIF-1 on the PEPCK (phosphoenolpyruvate carboxykinase) promoter under hypoxic conditions in hepatic cells [29]. In addition, other proteins in the AP-1 family were demonstrated to be involved in terms of gene regulation along with HIF-1 under hypoxic conditions [30].
The regulation of HIF-1α stability and activity occurs on multiple levels. In particular, the modulation of HIF-1α protein via the interaction between HIF-1α and other proteins is pivotal to the stabilization and activation of HIF-1α protein. In the present study, our results revealed that direct interaction with ATF-2 and HIF-1α contributes to the up-regulation of HIF-1α protein levels under hypoxic conditions. Along with hypoxia, the treatment of the hypoxia mimetic CoCl2 is well documented to increase levels of cellular ROS (reactive oxygen species) [31,32], and an increased ATF-2 protein level in superoxide-treated cells has been reported previously [33]; thus the increased level of cellular ROS induced by CoCl2 or hypoxia may be one of the reasons for the induction of ATF-2. The induction of ATF-2 was also shown to be an immediate event along with induction of HIF-1α under hypoxic stress conditions (Figure 1B).

Increased levels of ATF-2 under hypoxic conditions were not attributable either to increases in ATF-2 mRNA expression or to its stability (Figure 2A), but were ascribed to the prolonged half-life of the ATF-2 protein (Figures 2B and 2C). There are some reports asserting that ATF-2 is activated under hypoxic conditions [28], but thus far there have been no efforts to elucidate the relationship of ATF-2 with the master key regulator, HIF-1. The results we obtained using siRNA against HIF-1α showed that the induction of ATF-2 under hypoxic conditions is an HIF-1α-independent event. This result is consistent with the observations of another group, namely that one of the ATF-family proteins, ATF-4, was induced by anoxia via an increase in protein stability occurring in an HIF-1-independent manner [34]. Recently, Koditz et al. [35] also argued that induction of ATF-4 under anoxic conditions is mediated by reduced activity of HIF1-PHD3, which can hydroxylate ATF-4, leading to enhanced ATF-4 protein stability. The protein stability of ATF-2 is regulated by a ubiquitin-mediated proteasomal degradation mechanism [36], similar to that observed for other proteins. In the present study, cells that were exposed to three different conditions that can inhibit PHD activity (CoCl2 treatment, DP treatment and incubation under hypoxia) showed decreased ubiquitinated ATF-2 (Figure 2D). This result indicates that under hypoxic conditions, an unknown mechanism(s) involving PHD may enable the maintenance of ATF-2 in an activated state.

Many reports have asserted that members of the AP-1 family of transcription factors bind to cis-acting elements existing within the promoters of hypoxia-responsive genes, including those encoding VEGF, tyrosine hydroxylase and endothelin-1, where they potentiate their HIF-1-mediated hypoxic induction [37–39]. As a member of the AP-1 transcription-factor family, the possibility exists that ATF-2 can bind to these cis-elements, thus resulting in enhanced transcription of HIF-1 target genes. However, our results show that mutated ATF-2, which lacks its DNA-binding domain, also increases HIF-1α transcriptional activity (Figure 6C). This result reveals that the DNA binding activity of ATF-2 is not necessary for the regulation of HIF-1. Landazuri and colleagues [25] previously showed that c-Jun, the best-known heterodimer partner of ATF-2, can co-operate with HIF-1α via indirect interaction between two proteins, and they also demonstrated that the DNA binding of c-Jun on the AP-1 site was unnecessary for co-operation with HIF-1. In Figure 6(A), we demonstrated that ATF-2 can interact with HIF-1α under hypoxic conditions as well. Although many reports have thus far supported the notion that AP-1 proteins play a role in HIF-1α-mediated gene regulation, there is as yet no evidence of a direct physical interaction occurring between AP-1 and HIF-1α. Figure 6(B) shows that ATF-2 can bind directly to HIF-1α in vitro, thus showing that ATF-2 can act as a mediator of the co-operation of HIF-1 with other AP-1 proteins, including c-Jun. The induction of c-Jun expression by immediate-early signals in response to hypoxic...
conditions has been attributed, in part, to a positive-feedback loop in which (c-Jun/ATF-2)—AP-1 complexes bind to the c-Jun-proximal promoter region and stimulate transcription [40].

In the final step of our present study, we attempted to determine how ATF-2 can increase HIF-1α protein stability. The tumour suppressor proteins pVHL and p53 are known to be involved in the HIF-1α-degradation pathway, and thus the effect of ATF-2 overexpression on the interaction between HIF-1α and these proteins was assayed. The interaction of pVHL with HIF-1α, an interaction that is regulated by oxygen-sensor PHDs, was not altered by ATF-2 under either normoxic or hypoxic conditions (results not shown), whereas ATF-2 overexpression inhibits the binding of p53 to HIF-1α (Figure 7A), resulting in the blockage of p53-mediated degradation and the inhibition of HIF-1α (Figures 7B and 7C).

HIF-1α performs a pivotal role in the development of many types of cancer, principally by regulating the expression of target genes to enable adaptation under hypoxic stress in solid tumours. HIF-1α is up-regulated in the hypoxic regions of these tumours, but it can also be induced via the inactivation of particular tumour suppressor genes, including the VHL, p53 and PTEN (phosphatase and tensin homologue deleted on chromosome 10) genes, or by the action of particular oncogenes, including the v-SRC gene [5,6,41,42]. ATF-2 has been previously associated
with tumour growth and metastasis [43, 44] in both cell-culture and animal models. ATF-2 is involved in the transcriptional regulation of c-Jun as well as a number of cell-cycle genes, including cyclin A, cyclin D1 and ATF-3 [45–47]. In the present study, we have elucidated the distinct role of ATF-2 as a regulator of HIF-1α. Under hypoxic-stress conditions, the ATF-2 protein is induced, and, as a competitor of the p53 tumour suppressor, ATF-2 binds to HIF-1α protein and stabilizes it, resulting in the enhancement of HIF-1-mediated gene expression.

**AUTHOR CONTRIBUTION**

Jeong Hae Choi performed most of the experiments, Hyun Kook Cho assisted with some experiments, Yung Hyun Choi provided experimental materials, and Jaehun Cheong planned the entire study and wrote the manuscript.

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**REFERENCES**


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