

# HIF2 $\alpha$ -Sp1 interaction mediates a deacetylation-dependent *FVII*-gene activation under hypoxic conditions in ovarian cancer cells

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## ABSTRACT

Hypoxia-inducible factors (HIF)-1 $\alpha$  and HIF2 $\alpha$  are major transcription factors required for adaptive responses to hypoxia. HIFs form a complex with aryl hydrocarbon receptor nuclear translocator (ARNT) to bind to the regulatory regions of target genes. The acetylation of histones by histone acetyltransferases (HATs) is one of the epigenetic marks associated with active chromatin. Indeed, HIFs recruit p300 HAT to hypoxia response elements (HREs) within gene regulatory regions. Here, we report an unusual HIF-mediated transcriptional activation in ovarian clear cell carcinoma (CCC). While characterizing coagulation factor VII (*FVII*) gene induction during hypoxic conditions, we observed that the interaction of HIF2 $\alpha$  with Sp1, but not with ARNT, could induce transcription of *FVII* in a HRE-independent manner. Unexpectedly, this gene activation is associated with histone deacetylation. We found that a class II HDAC, HDAC4, is recruited with HIF2 $\alpha$  to the *FVII* promoter as a co-activator, while p300 HAT negatively regulated this process. Furthermore, this mechanism can be synergistically enhanced via a deacetylation-dependent pathway when cells are simultaneously exposed to hypoxic and serum-free conditions. These results suggest the presence of a stress-responsive transcription mediated by the HIF2 $\alpha$ /Sp1/HDAC4 network and explain how CCC shed their procoagulant activity under hypoxia.

## INTRODUCTION

The activation of various signaling pathways allows cells to adapt to be environmental stresses, such as O<sub>2</sub> and/or nutrient deprivation. Hypoxia inducible factors, HIF1 $\alpha$  and HIF2 $\alpha$ , are two of the main nuclear transcription factors that permit the appropriate cellular responses to reduced oxygen tension surrounding cancerous tissues (1). Under normal oxygen levels, or normoxia, HIF expression levels are very low because these factors undergo proteasomal degradation. However, under O<sub>2</sub>-deprived conditions, HIFs escape from ubiquitination by E3 ubiquitin ligases and accumulate within the cell. HIFs form heterodimeric complexes with the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT, also called as HIF1 $\beta$ ), enabling them to bind to hypoxia response element (HRE) sequences (1). This HIF-ARNT complex is associated with gene regulatory regions and functions to enhance transcription.

Histone acetylation is closely associated with transcriptional activation. Histone acetyltransferases (HATs) are recruited to regulatory regions of genes to catalyze the acetylation of histones and make chromatin structures more accessible for other transcriptional activators (2). Histone deacetylation, in contrast, is catalyzed by histone deacetylases (HDACs), which are associated with chromatin when transcription is down-regulated (2). One of the most important HATs, p300, is responsible for constitutive and inducible activation of numerous genes (2). Indeed, p300 is a transcriptional co-activator for HIFs, responsible for activating crucial events during cell survival, such as angiogenesis and metastasis (3).

Blood coagulation factor VII (fVII) is a key enzyme in the extrinsic coagulation cascade (4). Predominantly

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produced by hepatocytes, fVII interacts with its cellular receptor, tissue factor (TF) and, when bound to TF, is converted to its active form (fVIIa), thereby triggering a downstream coagulation cascade (5). This TF–fVIIa complex is known to initiate key pathogenic mechanisms in cancer, including cell motility, invasion, cell survival and angiogenesis (5), and is a major cause of thrombosis in cancer patients. In particular, in ovarian clear cell carcinoma (CCC), one of the most aggressive ovarian malignancies, the TF–fVIIa complex can cause venous thromboembolism (6), a common complication of cancer.

In CCC, fVII synthesis is induced in response to hypoxia (6,7), leading to the secretion of microvesicles that contain the TF–fVIIa complex; this secretion is likely to be one of the key causes of thrombosis in CCC-type patients with poor prognosis (6). We previously showed that various cancer cells could ectopically synthesize fVII (7). When this TF–fVIIa complex is derived from ectopically expressed fVII, cancer cells show enhanced motility and invasion (7). However, detailed mechanisms of the fVII induction are not clear. While we were characterizing the mechanism of hypoxic fVII-gene activation, we unexpectedly observed that HIF2 $\alpha$ , but not HIF1 $\alpha$ , binds to the *FVII* promoter region in the absence of HRE sequences (7), implicating that a novel cellular mechanism of adaptive response may exist. Thus, in this study, we sought to determine the detailed mechanisms of this unusual gene activation, including the involvement of histone acetylation, in an attempt to gain an understanding of the molecular mechanism of thrombosis in CCC patients. In addition, because cancer tissues with poor vascularization tend to be deficient in other serum factors in addition to molecular oxygen and nutrients (8), we further investigated how the deficiency of these factors affects this transcriptional regulation.

## MATERIALS AND METHODS

### Cell culture and reagents

Human cancer cell lines were maintained as previously described (7). Acriflavine (A8126) and tunicamycin (T7765) were purchased from Sigma (St Louis, MO, USA). Cell culture media used for experiments under glucose-free condition were from Invitrogen (11875 and 11879; Carlsbad, CA, USA).

### Expression vectors

Expression vectors of HIF1 $\alpha$ , HIF2 $\alpha$ , and p300 were prepared by PCR amplification using PrimeScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan) with the following primers: HIF1 $\alpha$ , 5'-CTAGCTAGCACCG ATTCACCATGGAGGGCG-3' (forward; F) and 5'-GG GGTACCGCTCAGTTAACTTGATCCAA-3' (reverse; R); HIF2 $\alpha$ , 5'-TCGACGCGTCGACAATGACAGCTG ACAAGGAG-3' (F) and 5'-GCTCTAGAGCTCAGGT GGCCTGGTCCAG-3' (R); p300, 5'-CCGCTCGAGCC TCGCTTGTATCTCCGAAAGAAT-3' (F) and 5'-GGG GTACCGGTGTCTCTAGTGTATGTCTAGTGTACT CTGT-3' (R). Fragments containing full-length cDNA derived from OVSAYO cells were inserted into the pCI

vector (Promega, Madison, WI, USA) at the *Nhe* I/*Kpn* I (HIF1 $\alpha$ ), *Mlu* I/*Xba* I (HIF2 $\alpha$ ), and *Xho* I/*Kpn* I (p300) sites.

### Small interference RNA experiments

Small interference RNAs (siRNAs) for HIFs were prepared as previously described (9). siRNAs for Sp1, p300, ARNT, and HDAC4 were ON-TARGET plus SMART pool reagents (Dharmacon, Lafayette, CA). Silencer Negative Control 1 RNAi (Ambion) was used as non-specific siRNA. Transfection was performed using lipofectamine RNAi MAX (Invitrogen).

### Quantitative RT-PCR analysis

We determined mRNA levels by quantitative RT-PCR, as previously described (7).

### Construction of luciferase plasmids and reporter gene assay

A luciferase plasmid construct with the *FVII* 5' full promoter region was prepared as previously described (10). Mutant constructs were prepared using the QuikChange mutagenesis Kit (Stratagene, La Jolla, CA). Luciferase assays were performed as previously described (10).

### Western blot analysis

Western blotting was performed as previously described (10). Antibodies used for western blotting or immunoprecipitation were Flag M2 (F1804, Sigma), HA (H6908, Sigma), HDAC1 (sc-7872, Santa Cruz Biotechnology, Santa Cruz, CA), HDAC2 (Sc-7899, Santa Cruz), HDAC3 (Sc-11417, Santa Cruz), HDAC4 (ab1437, Abcam, Cambridge or 2072S, Cell Signaling, Danvers, MA), SIRT1 (1104-1, EPITOMICS, Burlingame, CA), ARNT (NB100-110, Novus Biologicals, Littleton, CO), Sp1 (NB600-232, Novus Biologicals), and CHOP (2895, Cell Signaling).

### Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (10). The primers, probes and antibodies used are described elsewhere (7). Other antibodies used in this study were as follows: ARNT (NB100-110, Novus Biologicals), acetyl-histone H3 (06-599, Millipore, Temecula, CA), acetyl-histone H4 (06-866, Millipore), histone H3 (ab1791, Abcam), HDAC4 (ab1437, Abcam), HDAC-1 (sc-7872, Santa Cruz) and HDAC-2 (Sc-7899, Santa Cruz). Primers used for ChIP analysis of *VEGF*-HRE region by real-time PCR were 5'-GCCTCTGTCTGCCAGCTGC-3' and 5'-GTG GAGCTGAGAACGGGAAGC-3'; hybridization probes were 5'-TTGCCAGACTCCACAGTGCATACGTGG -FITC-3', and 5'-LCRed640-TCCAACAGGTCCTCTTC CCTCCAG-3'.

### Co-immunoprecipitation analysis

Expression vector of Sp1 tagged with a flag peptide at the N-terminus (pCI-Sp1-Flag) and an expression vector of HIF2 $\alpha$  tagged with a hemagglutinin peptide

(pCI-HIF2 $\alpha$ -HA) were prepared using QuikChange kit by inserting corresponding DNA sequences into the pCI-Sp1 and pCI-HIF2 $\alpha$  plasmids, respectively. Expression vectors of truncated HIF2 $\alpha$  were also prepared using QuikChange kit as deletion mutants of pCI-HIF2 $\alpha$ -HA. pCI-Sp1 was prepared by inserting the full-length cDNA fragment amplified from OVSAYO cells at the *XhoI/KpnI* cloning site. Primers used were 5'-CCGC TCGAGGACAGGACCCCTTGAGCTTG-3' (F) and 5'-GGGGTACCGGGGTATGGCCCATATGTC TCTG-3' (R). After transfection (24 h), cells were lysed with RIPA buffer (Sigma, R0278) and incubated with normal IgG, anti-Flag, or anti-HA antibodies (see "Western blot analysis" section) at 4°C for 2 h. Antibody-protein complexes were captured using Dynabeads-protein G (Invitrogen). Immunoprecipitates were analyzed by western blotting. Immunoprecipitation of endogenous Sp1 was performed using an anti-Sp1 antibody (NB600-232, Novus Biologicals) as described above.

## RESULTS

### HIFs mediate *FVII*-gene activation under hypoxia

We previously reported that the *FVII* gene is inducible in cancer cells under hypoxia-mimetic conditions created with CoCl<sub>2</sub> treatment (7), with highest *FVII* induction observed in the ovarian CCC cells, OVSAYO and OVISE. Thus, we used these cell lines to further examine the mechanism of the hypoxic gene activation. Using OVSAYO cells, we first performed a transcription factor-binding site analysis by inspecting an approximate 2500 bp region spanning an authentic *FVII* promoter and intron 1 (positions 3781–6180, GenBank accession number NG\_009262; <http://www.gene-regulation.com> and <http://www.cbrc.jp/research/db/TFSEARCHJ.html>). Our results identified the existence of candidate HREs upstream of the *FVII* 5' region (positions 4104–4109 and 4281–4286, accession number NG\_009262) close to predicted *Alu* elements (positions 4121–4417, accession number NG\_009262 and Figure 1A). However, ChIP analysis showed that HIFs did not bind to this region (Figure 1A and B, bar a) in response to CoCl<sub>2</sub> treatment (7) although both HIFs could occupy a HRE region of *VEGF* gene (Figure 1B). Unexpectedly, we found that HIF2 $\alpha$  principally bound to the *FVII* promoter region (Figure 1A, bar b) in the absence of a HRE site in OVSAYO cells (7), and to the *FVII* promoter under real hypoxic (1% O<sub>2</sub>) culture conditions (Figure 1B); this suggests that HIF2 $\alpha$  may be responsible for *FVII* gene induction. Finally, using western blotting and ChIP analysis, we confirmed the preferential binding of HIF2 $\alpha$  to the *FVII* promoter region in an additional CCC cell line, OVISE (Supplementary Figure S1A and S1B).

We next tested the effect of HIFs on *FVII* activation under CoCl<sub>2</sub> or 1% O<sub>2</sub> conditions. Using OVSAYO cells, we first suppressed HIF induction using HIF-specific RNA interference (RNAi) (9) and then examined its effect on *FVII* transcriptional induction using real-time RT-PCR analysis. We observed that *FVII* induction was

suppressed by the knock-down of not only HIF2 $\alpha$  but also HIF1 $\alpha$  (Figure 1C), as compared to non-specific (NS) siRNA-transfected experiments, and this could be further amplified by the concurrent knock-down of both HIFs (Figure 1C). In addition, quantitative ChIP analysis by real-time PCR supported our earlier findings that HIF1 $\alpha$  does not bind to the *FVII* promoter under hypoxia (Supplementary Figure S2A); this eliminated the possibility that trace amounts of HIF1 $\alpha$  up-regulated *FVII* transcription through association with the promoter region.

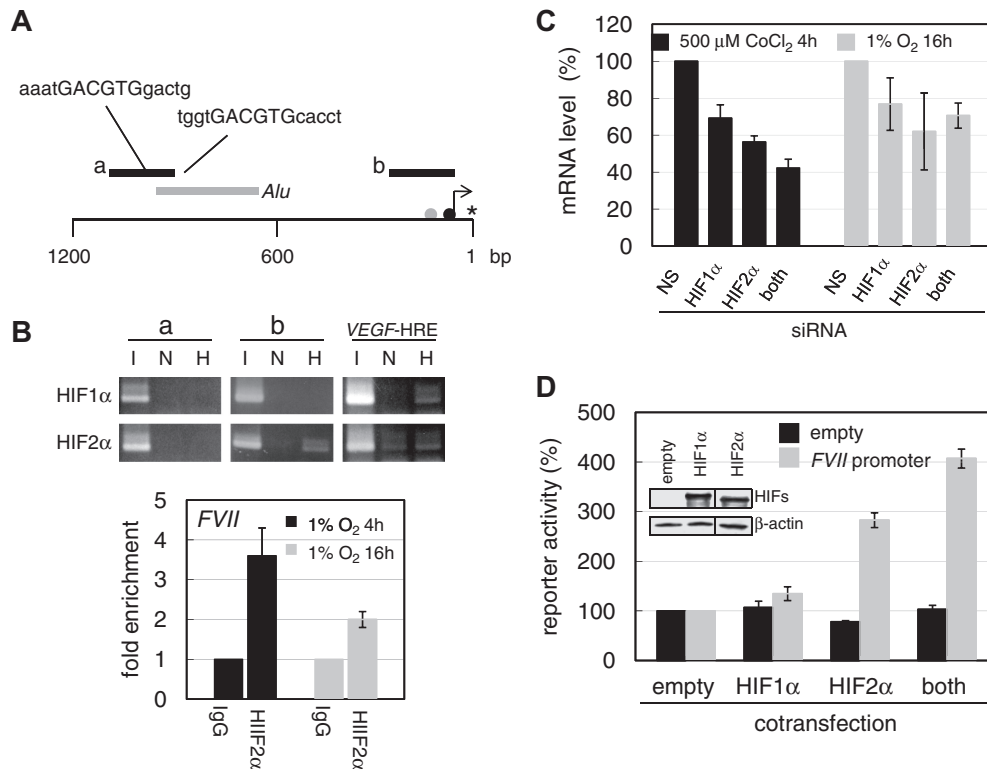
To investigate whether the *FVII* promoter region is activated by HIFs, we next performed a reporter gene analysis. A *FVII* fragment (–400/+1), which covers the full *FVII* promoter activity (10), was fused to a luciferase vector (Supplementary Figure S2B). The reporter assay revealed that the promoter was activated by ectopic expression of HIF2 $\alpha$ , whereas transfection of the same concentration (400 ng) of HIF1 $\alpha$  induced a very weak activation (Figure 1D). Interestingly, the promoter was most activated by the simultaneous expression of equal concentrations of both HIFs (200 ng each; Figure 1D). Positive control experiments with a HRE sequence of the *VEGF* gene (Supplementary Figure S2B) showed enhanced luciferase activity with HIF induction (Supplementary Figure S2C). As previously reported, HIF2 $\alpha$  caused the most activity, likely due to the differential regulation of HIF function and expression (11,12). Unlike with *FVII* activation, the activation of *VEGF* did not increase upon co-transfection of both HIFs. Together, these results suggest that HIFs may co-operatively activate the *FVII* promoter.

### An Sp1-binding site is responsible for activation of *FVII*

To further define the regions responsible for *FVII* activation, mutant luciferase constructs were co-transfected with both HIFs into OVSAYO cells. Constructs lacking a known Sp1-binding site demonstrated a weak response to the HIFs, similar to the response observed with the empty vector (Figure 2A). Using additional deletion constructs (Figure 2B), we found that promoter activation by HIFs was particularly impaired when the core Sp1-binding site of the *FVII* gene was mutated: the 5'-TCCT CCCCTCCCC-3' sequence (13) (Figure 2B; construct 4). Constructs with single or triple copies of this sequence responded primarily to HIF2 $\alpha$ , and when co-expressed with HIF2 $\alpha$ , HIF1 $\alpha$  co-operatively activated the luciferase expression (Figure 2C). Together, these findings suggest that the Sp1 site is responsible for *FVII* induction by HIFs.

### HIF2 $\alpha$ -Sp1 interaction induces *FVII* activation under hypoxia

We previously showed that Sp1 binds to the *FVII* promoter in OVSAYO cells (10). Thus, we next tested whether Sp1 is required for *FVII* induction under hypoxia-mimetic and real-hypoxic conditions. In OVSAYO cells, RNAi was first used to suppress Sp1 without affecting HIF protein levels (Figure 3A), before the cells were cultured with CoCl<sub>2</sub>. Real-time PCR



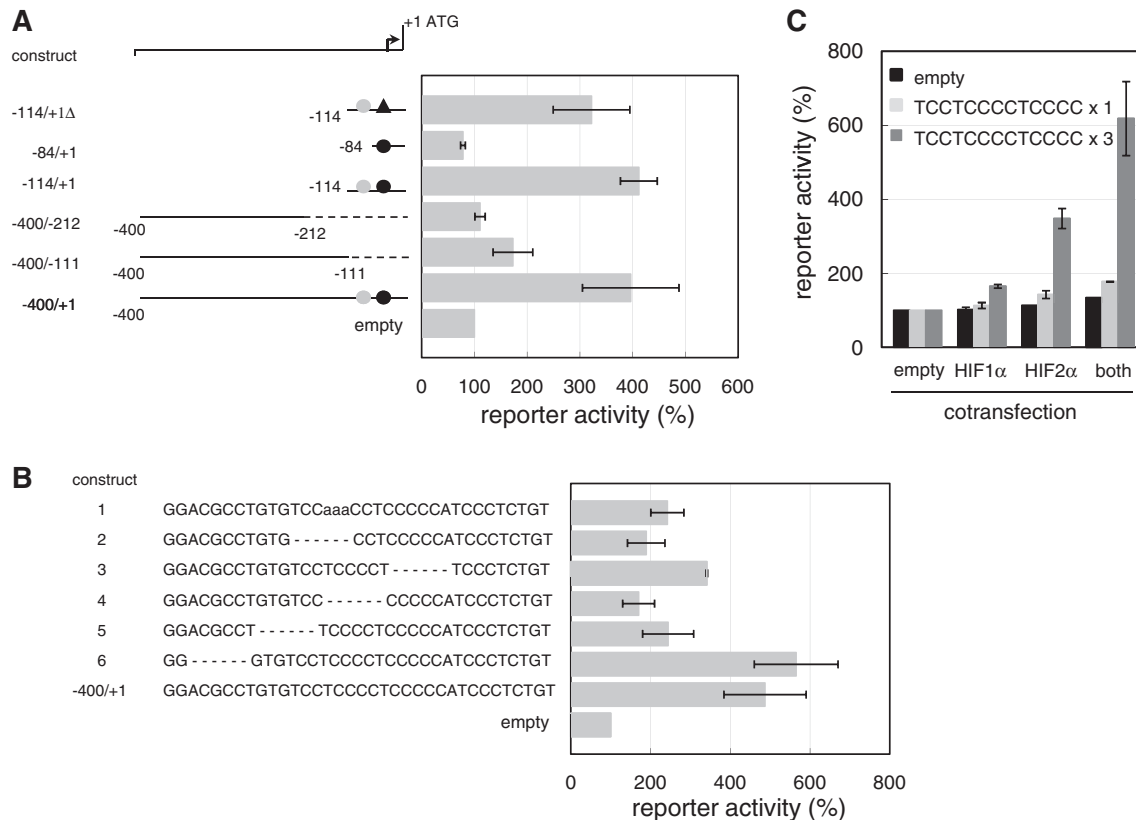
**Figure 1.** HIFs mediate *FVII* activation under hypoxia. (A) Schematic representation of *FVII* 5'-region. Black bars indicate the PCR amplicon for ChIP assay. Nucleotide sequence with capital letters represents the HRE sequence predicted by a computer search. Gray bar designates the position of predicted putative *Alu* repetitive elements. Gray and black circles indicate Sp1 and HNF-4 binding sites, respectively. A bent arrow and an asterisk indicate transcription and translation start sites, respectively, as previously determined (13). (B) ChIP analysis of HIFs. ChIP was performed for the *FVII* regions and a *VEGF*-HRE region in OVSAYO cells. PCR primers for *FVII* regions are as in Figure 1A. Primers for the *VEGF*-HRE region are as previously described (7). 'I', 'N', and 'H' indicate input sonicated DNA fragments without immunoprecipitation, normoxia, and 1% O<sub>2</sub> for 16h, respectively. Data were also estimated by real-time PCR. Fold enrichment of immunoprecipitated DNA for each transcription factor against background (templates due to non-specific binding with IgG) is shown. Data are mean of three independent experiments (hereafter  $n = 3$ )  $\pm$  SD. (C) Effect of HIFs on the expression of *FVII* in OVSAYO cells. Cells were cultured in 500  $\mu$ M CoCl<sub>2</sub> for 4h and 1% O<sub>2</sub> for 16h and analyzed with real-time RT-PCR. NS: non-specific. Data are the mean ( $n = 3$ )  $\pm$  SD. (D) Luciferase reporter analysis of *FVII* activation by HIFs. A *FVII*-promoter construct (Supplementary Figure S2B) was transfected into OVSAYO cells with each HIF separately (400 ng) or together (200 ng of each). Inset: western blotting analysis of HIFs expressions 24h post-transfection.  $\beta$ -actin shows equal protein loading. Relative promoter activities are shown as percentages of the activity from cells transfected with both empty vectors. Variations in transfection efficiency were corrected with a control *Renilla* luciferase vector. Data are the mean ( $n = 3$ )  $\pm$  SD.

analysis revealed that Sp1 knockdown markedly reduced *fVII* mRNA induction in both OVSAYO (Figure 3B) and OVISAYO cells (Supplementary Figure S2D), and this same Sp1 dependency was observed under real-hypoxic conditions (Supplementary Figure S3A). Induction of the *VEGF* gene was not significantly influenced by a decrease in Sp1 (Figure 3B), even though Sp1 binds to the *VEGF* promoter, and is essential for basal promoter activity (14). Following this, we tested whether HIF2 $\alpha$  binding to the *FVII* promoter is diminished in Sp1-siRNA-treated OVSAYO cells under CoCl<sub>2</sub> treatment. Using ChIP analysis, we observed a decrease in the binding of HIF2 $\alpha$  by Sp1-knockdown, although the binding of both HIFs to the *VEGF*-HRE region was not significantly altered (Figure 3C).

We next wanted to examine whether HIF2 $\alpha$  interacts with Sp1. Immunoprecipitation analysis revealed that HIF2 $\alpha$  induced in OVSAYO cells cultured under 1% O<sub>2</sub> for 16h can be precipitated with endogenous Sp1 (Figure 4A); this suggests that Sp1 and HIF2 $\alpha$  interact

to form a protein complex. Furthermore, HA-tagged HIF2 and Flag-tagged Sp1 were co-transfected into OVSAYO cells (Figure 4B), and immunoprecipitated with anti-HA or anti-Flag antibodies. Western blotting results showed that HIF2 $\alpha$ -HA formed a protein complex with Sp1-Flag in OVSAYO cells (Figure 4C). To determine the binding sites, we then co-transfected various deleted mutants of HIF2 $\alpha$  (Figure 4D) with Sp1-Flag. We observed that Sp1 binds to HIF2 $\alpha$ , even in the absence of the HLH (90–870) and PAS-A (161–870) domains (Figure 4D and E), and interacts with the C-terminal region that includes the CTAD domain (551–870) of HIF2 $\alpha$ , but not when this portion was fused with the middle region containing the NTAD domain. Sp1 also associated with the HLH-PAS-A portion of the protein (1–201) as well as both PAS domains (90–351), but not with the HLH domain alone.

To verify the Sp1-dependent binding, we next performed ChIP analysis with an anti-HIF2 $\alpha$  antibody on OVSAYO cells transfected with luciferase constructs and



**Figure 2.** An Sp1 binding sequence is responsible for *FVII* activation by HIFs. (A) OVSAYO cells were co-transfected with *FVII*-promoter derived constructs and HIFs. Luciferase activity was estimated 24 h post-transfection. Gray and black circles indicate Sp1 and HNF-4 binding sites, respectively. Black triangles indicate a six base pair deletion of core HNF-4 binding sequence (10). (B) Construct with a region corresponding to -85 to -114 of the *FVII* promoter (Figure 2A), or its deletion mutants, were co-transfected with HIFs, and assessed for luciferase activity 24 h post-transfection. (C) Constructs inserted with single or triple copies of a Sp1-binding sequence (Figure 2B) were transfected with HIFs, and tested for luciferase activity after 24 h. For all experiments, relative promoter activities are shown as Figure 1D. Data are the mean ( $n = 3$ )  $\pm$  SD.

treated with  $\text{CoCl}_2$  (Supplementary Figure S3B). PCR analyses revealed that HIF2 $\alpha$  binds to the full-length promoter, and that binding can be abrogated upon deletion of the Sp1 site. In addition,  $\text{CoCl}_2$  treatment did not affect the expression (6) or binding levels (Supplementary Figure S3C) of Sp1 to the *FVII* promoter, suggesting that HIF2 $\alpha$  interacts with Sp1 that is constitutively associated with promoter region, and that HIF2 $\alpha$  does not activate the *FVII* promoter by increasing Sp1 binding.

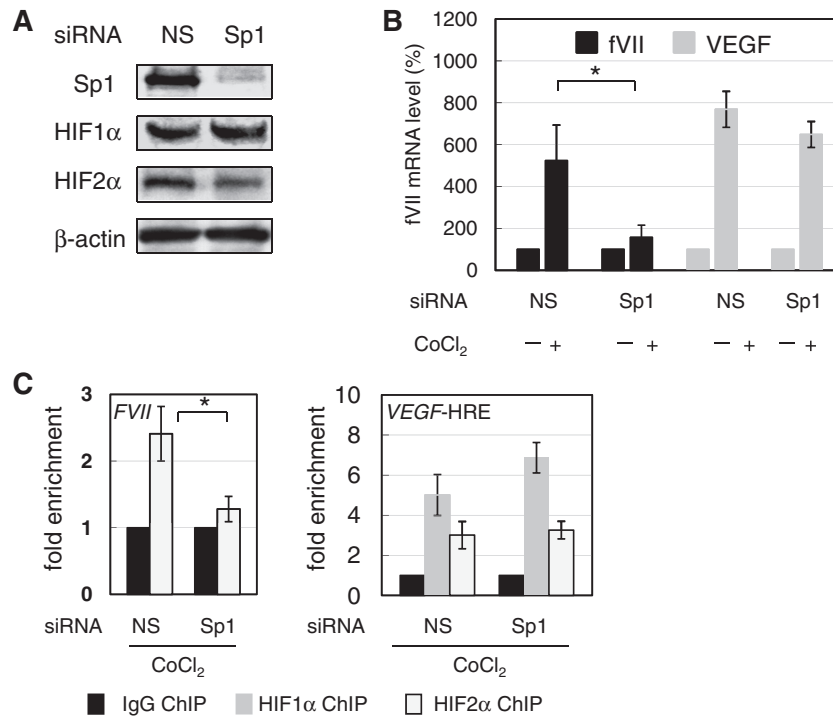
#### *FVII* activation does not require ARNT

HIFs induced under hypoxia form heterodimeric complexes with constitutively expressed ARNT to bind HRE sequences. Thus, we next sought to determine whether this complex formation is necessary for *FVII* activation. CHIP analysis revealed that ARNT did not bind to the *FVII* promoter in response to  $\text{CoCl}_2$  stimulation (Figure 5A); however, ARNT was recruited to the *VEGF*-HRE site under these conditions (Figure 5A). Following this, we assessed the effect of ARNT expression on *FVII* and *VEGF* gene inductions. Using RNAi, we first suppressed ARNT in OVSAYO cells (Figure 5B), in which induction of HIFs was not affected (Figure 5B). Using

real-time RT-PCR analysis, we detected a decrease and an increase in *fVII* and *VEGF* mRNA levels, respectively, when ARNT-suppressed cells were cultured under normoxia (Figure 5C). However, there was a marked reduction in *VEGF* mRNA when OVSAYO cells were treated with  $\text{CoCl}_2$  or 1%  $\text{O}_2$  with no change in the percentage of *fVII* mRNA as compared with the negative control (Figure 5C). Finally, we tested whether inhibiting the formation of the HIF-ARNT complex influences *FVII* activation. OVSAYO cells were cultured with  $\text{CoCl}_2$  for 4 h in the presence of a heterodimerization inhibitor, acriflavine (ACF) (15). Real-time RT-PCR analysis showed that ACF treatment increased the *fVII* mRNA level via unknown mechanism (Supplementary Figure S4A), and reduced the mRNA levels of the HRE-dependent *VEGF* and *ENO1*, as expected (Supplementary Figure S4A).

#### Hypoxic *FVII* activation associates with deacetylation

HIFs recruit p300 HAT to HREs and activate target genes. Therefore, we wanted to assess how histone acetylation is linked to *FVII* activation. First, we tested the effect of  $\text{CoCl}_2$  treatment on the acetylation of histones H3 and H4 within the *FVII* promoter region in OVSAYO



**Figure 3.** HIF2 $\alpha$  binds *FVII* promoter via interaction with Sp1. (A) Effect of Sp1 knockdown on the expression of HIFs induced in OVSAO cells cultured in 500  $\mu$ M CoCl<sub>2</sub> for 4 h. (B) Effect of Sp1 on *FVII* expression in OVSAO cells cultured with or without 500  $\mu$ M CoCl<sub>2</sub> for 4 h after Sp1 knockdown. *FVII* expression was examined by real-time RT-PCR. *VEGF* expression was performed as a control of HRE-dependent expression. NS: non-specific. Data are the mean ( $n = 3$ )  $\pm$  SD. \* $P < 0.05$  (two-sided Student's  $t$ -test). (C) Effect of Sp1 on binding of HIFs to *FVII* promoter in OVSAO cells cultured in 500  $\mu$ M CoCl<sub>2</sub> for 4 h after Sp1 knockdown. ChIP analysis for HIFs binding was performed for *VEGF*-HRE and *FVII* regions. Data were estimated by qPCR. Data are the mean ( $n = 3$ )  $\pm$  SD.

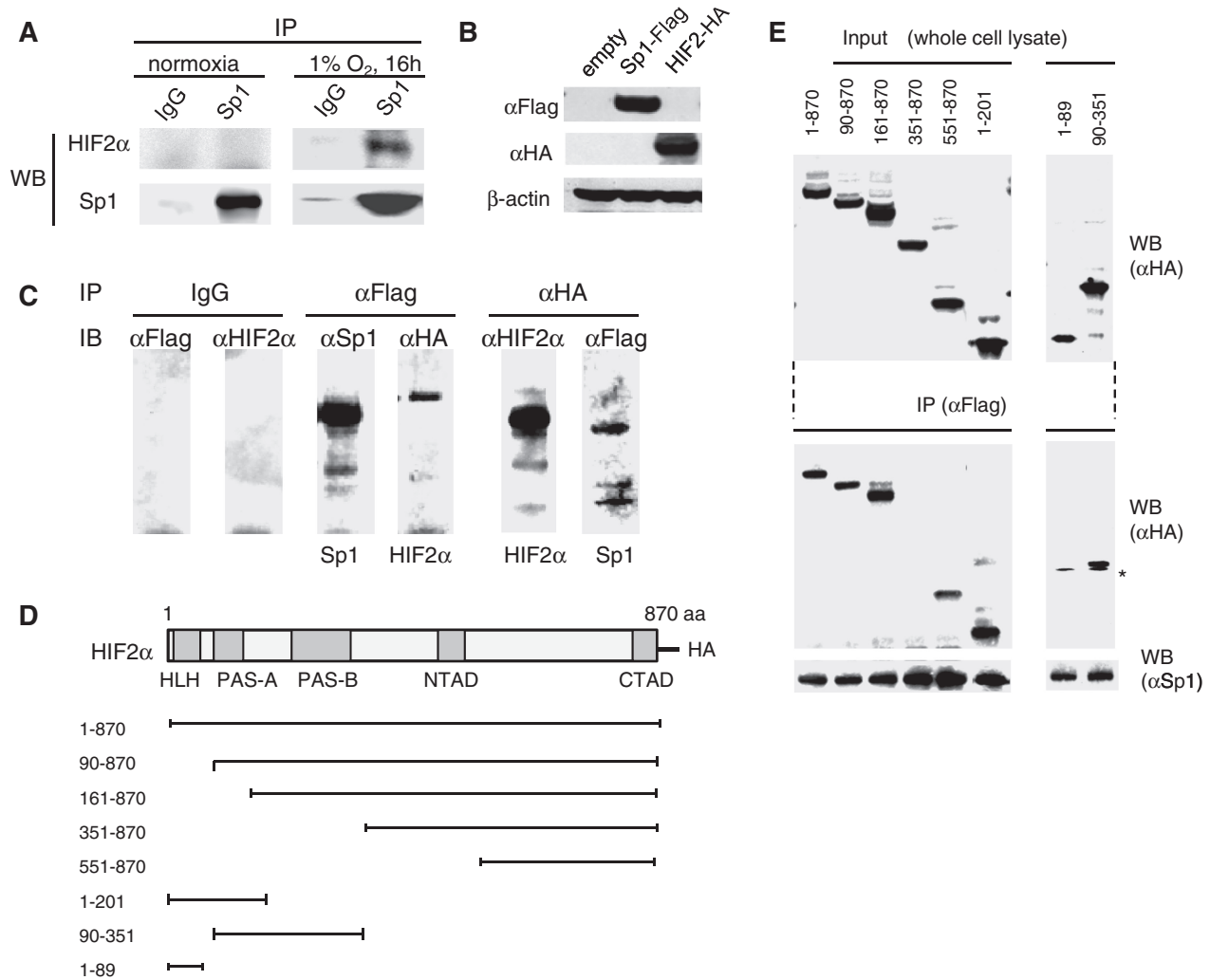
cells. As expected, ChIP analysis of *VEGF*-HRE region showed an elevation in the histone acetylation levels (Figure 6A, left and Supplementary Figure S4B). However, we unexpectedly found that histones within the *FVII* promoter were deacetylated in response to CoCl<sub>2</sub> treatment (Figure 6A, middle and Supplementary Figure S4B). ChIP analysis also showed that the association level of histone H3 within the *FVII* promoter was unchanged following CoCl<sub>2</sub> stimuli (Figure 6A, right), further verifying that decreased acetylation levels did not result from histone eviction. We also confirmed that histones were deacetylated within the *FVII* promoter under CoCl<sub>2</sub> treatment in OVISE cells (Supplementary Figure S4C), and under real hypoxic conditions in OVSAO cells (Figure 6A, middle).

Steady-state histone acetylation across chromatin is maintained by the dynamic equilibrium between HATs and HDACs (2,16). Although we observed p300 binding to the *FVII* promoter in OVSAO cells, the association level was not altered after CoCl<sub>2</sub> treatment (Figure 6B). Conversely, as expected, p300 binding to *VEGF*-HRE was elevated by CoCl<sub>2</sub> treatment (Figure 6B). Since hypoxic *FVII* activation is associated with histone deacetylation, we tested the role of p300 on this transcriptional activation. Using RNAi, p300 was first suppressed (Figure 6C), before the cells were cultured under 1% O<sub>2</sub> for 16 h. Using real-time RT-PCR, we observed an increase in the *fVII* transcript level with p300-knockdown, whereas *VEGF*

transcript levels were diminished (Figure 6D). Furthermore, ectopic expression of p300 suppressed *FVII* promoter activation when HIF2 $\alpha$  alone or both HIFs were expressed (Figure 6E). In contrast, there was a relatively low increase in *VEGF*-HRE activation by HIF1 with ectopic p300 expression, similar to the level observed by HIF2 expression (Figure 6E). These results demonstrate that deacetylation promotes *FVII* activation by HIFs.

#### HDAC4 is a co-activator of *FVII* induction

As HRE-dependent mechanisms recruit HAT to activate transcription, we surmised that HDACs may co-activate *FVII* under hypoxic conditions. Thus, we first examined recruitment of the ubiquitously expressed HDAC1 and HDAC2 to the *FVII* promoter region in response to CoCl<sub>2</sub> treatment. ChIP analysis revealed no change in the association levels of HDAC1 and HDAC2 after CoCl<sub>2</sub> exposure (Supplementary Figure S5A). A recent report found that SIRT1 deacetylated HIF2 $\alpha$  (12); therefore, we tested whether class III HDAC(s) contributed to *FVII* induction. Whilst ChIP analysis revealed a weak increase in SIRT1 levels (Supplementary Figure S5B) and some binding to the *FVII* promoter in OVSAO cells, this was not enhanced by CoCl<sub>2</sub> stimulation (Supplementary Figure S5C). In addition, although SIRT1 associated with HRE of the *VEGF* gene, the binding was abrogated with CoCl<sub>2</sub> (Supplementary



**Figure 4.** (A) Co-immunoprecipitation analysis of endogenous Sp1 and HIF2 $\alpha$  expressed in OVSAYO cells. (B) Western blot analysis of Flag-Sp1 and HA-HIF2 $\alpha$  expressed in OVSAYO cells. (C) HIF2 $\alpha$  interacts with Sp1 in OVSAYO cells. Cells transfected with HIF2-HA and Sp1-Flag were subjected to immunoprecipitation 24 h post-transfection and analyzed by immunoblotting. (D) Truncated HIF2 $\alpha$  proteins used for co-immunoprecipitation experiments. (E) Cells were transfected with truncated HIF2-HAs and Sp1-Flag and immunoprecipitated with anti-Flag antibody 24 h post-transfection. An asterisk indicates a non-specific band.

Figure S5C). Similarly, the class III HDAC activator, resveratrol (12), did not promote *FVII* activation under CoCl<sub>2</sub> treatment (Supplementary Figure S5D).

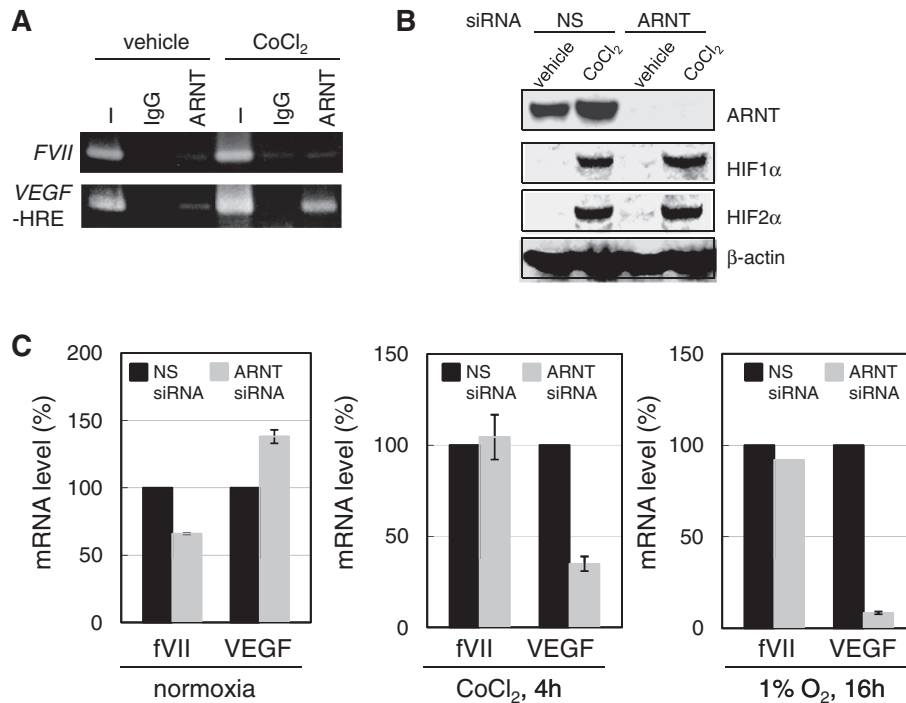
We next examined whether class II HDAC(s) (17) affect *FVII* activation. Western blotting showed that the class II HDAC, HDAC4 (Figure 7B) rather than HDAC5 (Supplementary Figure S5E), was predominantly expressed in OVSAYO cells. ChIP analysis showed the recruitment of HDAC4 to the *FVII* promoter in response to CoCl<sub>2</sub> treatment or hypoxia (Figure 7A). Real-time RT-PCR analysis revealed that HDAC4-knockdown does not affect basal *fVII* transcript levels in OVSAYO cells (Figure 7C); however, we observed a significant reduction in its induction with CoCl<sub>2</sub> treatment (Figure 7D) without influencing HIF expression levels (Figure 7B) or HIF2 $\alpha$  binding to *FVII* promoter (Figure 7E). In addition, immunoprecipitation analysis revealed an association between HDAC4 and endogenous

Sp1-HIF2 $\alpha$  complex under hypoxia (Supplementary Figure S5F).

We also tested whether HDAC3 binds to the *FVII* promoter in response to CoCl<sub>2</sub> treatment. Recently, it was shown that HDAC4 recruits and activates the transcription factor, FOXO, via deacetylation by HDAC3 (18). Although HDAC3 is expressed in OVSAYO cells (Supplementary Figure S5B), we only observed weak binding of HDAC3 to the *FVII* promoter region, which was not enhanced with CoCl<sub>2</sub> treatment (Supplementary Figure S5G). In comparison, HDAC3 showed a strong interaction with HRE of the *VEGF* gene in OVSAYO cells (Supplementary Figure S5G).

**Serum deprivation but not glucose deprivation synergistically enhances *FVII* activation under hypoxia**

Cancer cells must adapt to environmental stress conditions, particularly in cases where inefficient vascularization



**Figure 5.** (A) ChIP for ARNT binding was performed for *FVII* and *VEGF*-HRE regions in OVSAYO cells cultured in the presence or absence of CoCl<sub>2</sub>. (B) Western blotting of ARNT expression in cells transfected with NS- or ARNT-siRNA. (C) Effect of ARNT on the expression of *FVII* and *VEGF* in OVSAYO cells cultured under normoxia, 500 μM CoCl<sub>2</sub> for 4 h, or 1% O<sub>2</sub> for 16 h. After ARNT knockdown, cells were cultured under above conditions. Real-time RT-PCR analysis was performed. Data are the mean ( $n = 2$ ) ± SD.

within the tissue leads to O<sub>2</sub> and nutrient deprivation (8). Thus, we first tested how glucose deprivation influences *FVII*-gene activation under hypoxia. Real-time RT-PCR analysis revealed that although glucose deprivation weakly increased fVII mRNA levels, the transcript levels were not further enhanced when cells were simultaneously exposed to 1% O<sub>2</sub> (Supplementary Figure S6A). These results are consistent with data showing decreased HIF expression in cancer cells under glucose-free conditions (19) and the weak expression of HIF observed in OVSAYO cells under glucose-deprived conditions (Supplementary Figure S7B).

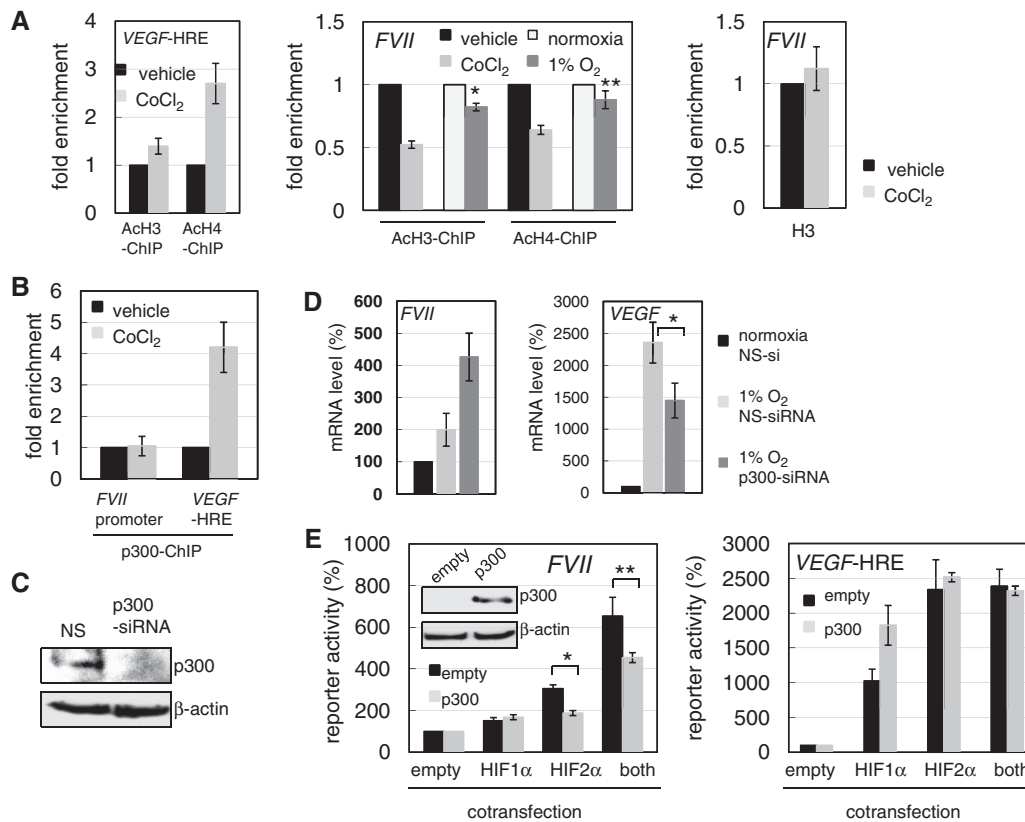
Serum starvation has been reported to induce a cellular response to hypoxia through the hypoxia-inducible factor-independent pathway (20). Hypoxia and serum deprivation are known to be components of ischemia (21,22), and we have previously demonstrated that CCC cells efficiently shed TF-fVIIa-containing microvesicles under both hypoxic and serum-free conditions (6). Since hypoxic cancer tissues should also be devoid of serum factors other than O<sub>2</sub> and nutrients, we next examined whether serum starvation affects *FVII* activation. Cells were cultured with or without fetal calf serum (hereafter serum) under normoxia or 1% O<sub>2</sub>. Real-time RT-PCR analysis revealed a slight increase in fVII transcript levels in serum-deprived cells cultured under normoxia (Figure 8A); this is possibly owing to a weak induction of HIFs (Supplementary Figure S6B). However, fVII was synergistically increased at both the mRNA and protein levels when cells were exposed to both hypoxic and

serum-free conditions (Figure 8A and B). In contrast, the VEGF mRNA levels were not significantly influenced (Figure 8A). ChIP analysis showed that HIF2α predominantly binds to the *FVII* promoter (Figure 8C), similar to that observed in serum-plus conditions (Figure 1B); this eliminated the possibility that an altered binding pattern of HIFs caused this synergistic activation. Furthermore, the association of Egr-1 with the *FVII* promoter under normoxia expression was unchanged by exposing the cells to hypoxic and serum-free conditions (Supplementary Figure S6C); Egr-1 is a transcription factor responsible for hypoxic activation of tissue factor gene by substituting Sp1 binding (23). Knockdown of p300 and HDAC4 increased and decreased hypoxic fVII mRNA induction, respectively, under serum-free conditions (Figure 8D). Furthermore, the acetylation level of histones within the *FVII* promoter was diminished under both hypoxic and serum-free conditions (Figure 8E), as in the case of CoCl<sub>2</sub> treatment, demonstrating a synergistic activation associated with deacetylation.

#### UPR signaling is not responsible for the synergistic transcriptional activation

Other than mechanisms mediated by HIFs, endoplasmic reticulum (ER) stress is a common cellular stress induced by stimuli, such as O<sub>2</sub> and nutrient deprivation (24,25). Cells are able to adapt to such environments via unfolded protein response (UPR), which is mediated by the induction of various stress response proteins, such as CHOP, and can co-operate with HIF signaling.





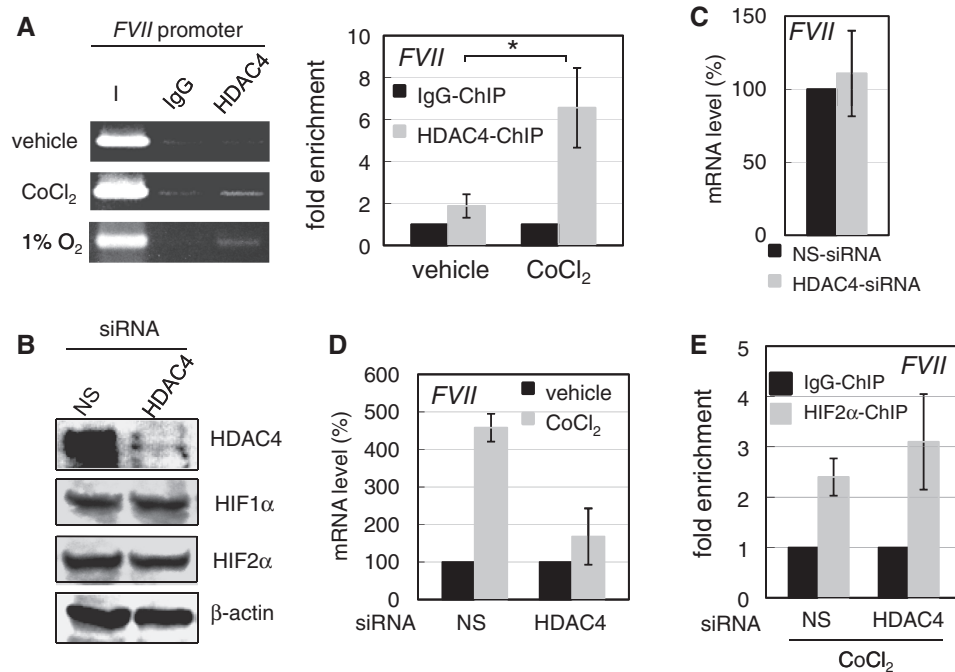
**Figure 6.** Hypoxic *FVII* activation associates with deacetylation. (A) Effect of CoCl<sub>2</sub> treatment or hypoxia on histone acetylation at *VEGF*-HRE and *FVII* loci in OVSAYO cells. ChIP analysis for acetylated histones (AcH3 and AcH4) and histone H3 was performed by real-time PCR. Data are the mean ( $n = 3$ )  $\pm$  SD. \* $P < 0.005$ , \*\* $P < 0.08$ , compared with normoxia control. (B) p300 binding was analyzed by quantitative ChIP analysis for *FVII* and *VEGF*-HRE loci in OVSAYO cells treated with CoCl<sub>2</sub> for 4 h. (C) Western blotting of p300 expression in OVSAYO cells transfected with NS- or p300-siRNA. (D) Effect of p300 on *FVII* and *VEGF* expressions in OVSAYO cells cultured under hypoxia. After p300 knockdown, cells were cultured under normoxia or 1% O<sub>2</sub> for 16 h. Real-time RT-PCR analysis was then performed. Data are the mean ( $n = 3$ )  $\pm$  SD. \* $P < 0.05$ . (E) Effect of p300 over-expression on activation of the *FVII* promoter and *VEGF*-HRE by HIFs. OVSAYO cells were transfected with a *FVII*-promoter construct, HIFs and p300, and examined by luciferase activity. Inset: western blotting analysis of p300 expression 24 h post-transfection. Relative activities are shown as a percentage of empty vector expression. Data were normalized to *Renilla* luciferase activity. Data are the mean ( $n = 3$ )  $\pm$  SD. \* $P < 0.005$ , \*\* $P < 0.05$ .

We hypothesized that UPR contributes to the synergistic *FVII* activation. We first examined whether cells cultured under our experimental conditions undergo ER stress. Western blotting showed that ER stress via CHOP induction only occurred when OVSAYO cells were exposed to both hypoxic and serum-free conditions (Figure 8F). We next tested whether UPR causes synergistic *FVII* induction. OVSAYO cells were cultured in the presence of an ER stress-inducing agent, tunicamycin (Tun) (26), in the presence of serum. The results confirmed that CHOP is induced by Tun treatment (Figure 8G). We next examined the effect of Tun on *FVII* expression under hypoxia. Real-time RT-PCR analysis revealed that, although Tun treatment elevated *FVII* transcript level under normoxia, it was not further up-regulated by hypoxia (Figure 8H). In addition, HIF levels induced under 1% O<sub>2</sub> were largely unchanged by simultaneous Tun exposure (Figure 8I); these results suggest that UPR is not involved in the synergistic activation. This result is consistent with data showing that glucose deprivation under normoxia weakly enhanced *FVII* activation (Supplementary Figure

S6A) with induction of CHOP (Supplementary Figure S7A), as UPR is known to be induced in cancer cells cultured under glucose-free condition (26).

## DISCUSSION

Hypoxic activation of the HRE-dependent *CAIX* (27), *RORA* (28), and *G3PDH* (29) by HIF1 $\alpha$  is modulated by Sp1 binding near the HRE. In addition, HIFs have been shown to interfere with the formation of the c-Myc/Sp1 and c-Myc/Max complexes that regulate gene expressions necessary for cell cycle (30) and mismatch repair (31) controls. Thus, Sp1 is generally considered to be an important regulator of gene expression under hypoxic conditions. Here, we showed a novel transcriptional activation of *FVII* mediated by HIFs that involves (i) the interaction of HIF2 $\alpha$  with Sp1, but not with ARNT; (ii) an association with histone deacetylation; and (iii) a synergistic activation under serum-free conditions. Furthermore, it is plausible that genes potentially regulated by this mechanism may be important in the



**Figure 7.** HDAC4 is critical for *FVII* activation under hypoxia and its mimetic conditions. (A) ChIP analysis of HDAC4 binding was performed for cells cultured with vehicle, 500  $\mu$ M CoCl<sub>2</sub> for 4 h, or under 1% O<sub>2</sub> for 16 h. ChIP was also performed by real-time PCR for cells cultured with vehicle or 500  $\mu$ M CoCl<sub>2</sub> for 4 h. Data are the mean ( $n = 3$ )  $\pm$  SD. \* $P < 0.05$ . (B) Western blotting of HDAC4 expression in OVSAYO cells transfected with NS- or HDAC4-siRNA. Effect of HDAC4 knockdown on the expression of HIFs induced by CoCl<sub>2</sub> treatment (500  $\mu$ M for 4 h) is also shown. (C) Effect of HDAC4 on *FVII* expression in cells cultured under normoxia. After HDAC4 knockdown, real-time RT-PCR was performed. Data are the mean ( $n = 3$ )  $\pm$  SD. (D) Effect of HDAC4 on *FVII* expression in OVSAYO cells cultured with CoCl<sub>2</sub>. After HDAC4 knockdown, cells were cultured with 500  $\mu$ M CoCl<sub>2</sub> for 4 h and analyzed by real-time RT-PCR. Data are the mean ( $n = 3$ )  $\pm$  SD. (E) Effect of HDAC4 knockdown on binding of HIF2 $\alpha$  to the *FVII* promoter in OVSAYO cells cultured in 500  $\mu$ M CoCl<sub>2</sub> for 4 h. ChIP analysis for HIF2 $\alpha$  binding was performed for the *FVII* promoter. Data were estimated by qPCR. Data are the mean ( $n = 3$ )  $\pm$  SD.

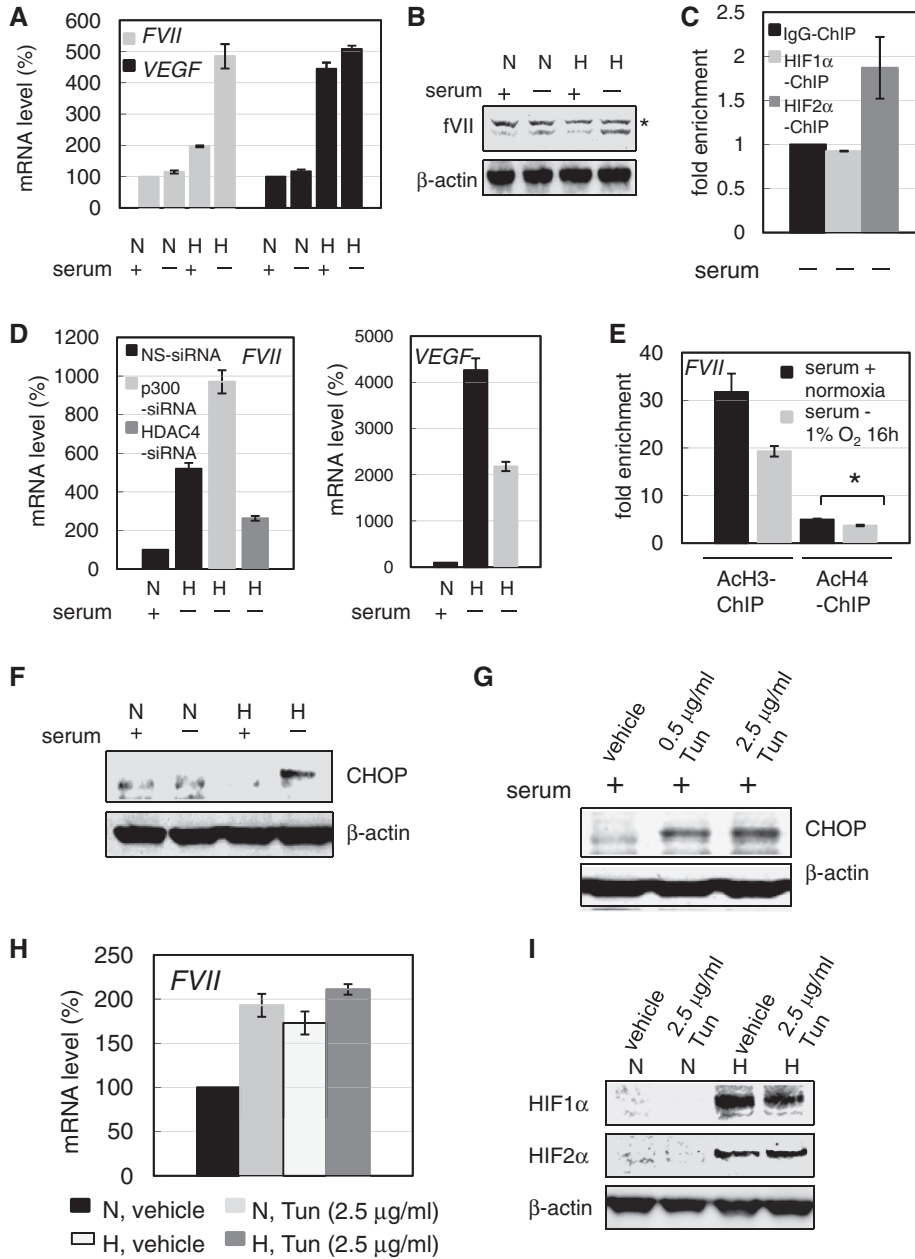
cellular response to severe hypoxic conditions with a poor supply of serum factor(s). Together, these findings have provided us with some explanation as to why CCC cells cultured under hypoxia/serum starvation can efficiently shed microvesicles enriched with *FVIIa* activity.

HIFs bind with ARNT through PAS domains at their N-terminus (32), whereas the C-terminus interacts with other transcriptional regulators, such as p300 and SIRT1 (33). In this study, we found that both ends of HIF2 $\alpha$  can associate with Sp1; however, further studies are required to clarify the mechanisms of the interaction between Sp1 and the C-terminus of HIF2 $\alpha$ . It was reported that cysteine (C)/histidine (H)-rich zinc finger domain of a Sp1/KLF family protein can bind to p300/CBP (34). Given that CTAD domain of HIF1 $\alpha$  can associate with a C/H-rich zinc-binding motif (CH1) of p300/CBP (35), it seems likely that zinc-finger domain of Sp1 binds to the C-terminus of HIF2 $\alpha$  through interaction with CTAD. However, the binding at the C-terminal region is abrogated by the presence of the middle region that contains the NTAD domain; this suggests that HIF2 $\alpha$  has an inhibitory domain that affects the interaction between the C-terminal region of HIF2 $\alpha$  and Sp1. It would be interesting to ascertain whether this inhibitory effect participates in the physiological regulation of HIF2 $\alpha$  function. In addition, we also observed that the HLH region alone does not associate with Sp1. From

these findings, we suggest that full-length HIF2 $\alpha$  binds to Sp1 through its PAS domains. This is similar to a previous report showing that PAS domains of ARNT can associate with zinc-finger domain of Sp1 (36). Assuming that HIF2 $\alpha$  binds with ARNT through its PAS domains, our data may explain why ARNT is not recruited with HIF2 $\alpha$  to the *FVII* promoter under hypoxia.

Notably, the mechanism described in this study is similar to that of constitutive *FVII* expression under normoxia in breast cancer cells (10), where the same Sp1-binding site is critical for activation, and HIFs co-operatively activate transcription. We suggest that HIF1 $\alpha$  may indirectly activate the *FVII* promoter region, as HIF1 $\alpha$  does not directly bind to the *FVII* promoter, yet demonstrates co-operative effects when simultaneously expressed with HIF2 $\alpha$ . The present mechanism is distinct from that of *ABCC8*-gene activation under hypoxia, in which there is a HIF1 $\alpha$ -mediated up-regulation of Sp1 (37). This is interesting, as we showed that the expression and binding levels of Sp1 to the *FVII* promoter under normoxia in OVSAYO cells were not influenced by CoCl<sub>2</sub> and hypoxia treatments.

Our finding that *FVII* activation associates with deacetylation was unexpected, because under normal circumstances, transcriptional activation closely associates with hyperacetylation of histones, not deacetylation.



**Figure 8.** HDAC4 but not UPR is critical for synergistic *FVII* activation induced under both hypoxia and serum-free conditions. (A) Effect of serum deprivation on *FVII* and *VEGF* expressions in OVSAYO cells cultured under normoxia (N) or hypoxia (H; 1% O<sub>2</sub> for 16 h). Results were measured by real-time RT-PCR analysis. Data are the mean (n = 3) ± SD. (B) Effect of serum deprivation on fVII expressions in OVSAYO cells cultured under normoxia or hypoxia as in (A). Asterisk indicates suspected non-specific background. (C) ChIP of HIFs binding to the *FVII* promoter in OVSAYO cells cultured under both hypoxia and serum-free conditions, performed using real-time PCR. Data are the mean (n = 3) ± SD. (D) Effect of p300 or HDAC4 on *FVII* and *VEGF* expression in OVSAYO cells cultured under both hypoxia and serum-free conditions. After 30 h of siRNA transfection, cells were further cultured for 16 h, as indicated, and analyzed by real-time RT-PCR. Data are the mean (n = 3) ± SD. (E) Decreased histone acetylation at the *FVII* promoter in OVSAYO cells cultured under both hypoxia and serum-free conditions. ChIP analysis was performed by real-time PCR. Data are the mean (n = 3) ± SD. \*P < 0.05. (F) CHOP induction in OVSAYO cells. Cells were cultured under normoxia (N) or hypoxia (H; 1% O<sub>2</sub> for 16 h) with or without serum for 16 h, and analyzed by western blotting. (G) Western blotting of CHOP induction in OVSAYO cells cultured with tunicamycin (Tun) in serum-plus medium for 16 h. (H) Effect of Tun on the expression of *FVII* in OVSAYO cells cultured under hypoxia in serum-plus medium via real-time RT-PCR. Data are the mean (n = 3) ± SD. (I) Effect of Tun on the expression of HIFs in OVSAYO cells cultured under hypoxia.

Indeed, previous work shows that basal *FVII* transcription is dependent on histone acetylation, with enhanced fVII mRNA levels in response to treatment of cancer cells with the HDAC inhibitor, trichostatin (10). Our data, showing that the expressions of p300 and HDAC4

correlate with a decrease and an increase in fVII transcript levels, respectively, suggests that the association between histone deacetylation and HIF2α recruitment may be a critical step for the formation of active chromatin.

Although this study has focused on the acetylation status of histones, it is also possible that deacetylation of HIF2 $\alpha$  by HDAC4 may affect its function in the regulation of *FVII* transcription in CCC cells, since acetylation of lysine residues is critical to the functional regulation of HIF (33). However, it was recently reported in cancer cells that HDAC4 causes deacetylation of HIF1 $\alpha$ , but not HIF2 $\alpha$ , to enhance its stability (38). Since HIF1 $\alpha$  participates in *FVII* activation in CCC cells, HDAC4 may affect *FVII* induction via modulation of HIF1 $\alpha$ . Furthermore, HDAC4 was recently shown to deacetylate the transcription factor, FOXO, in liver cells to enhance its binding activity to target genes required for gluconeogenesis (18,39). However, it is unlikely that HDAC4 recruitment contributes to the interaction between HIF2 $\alpha$  and Sp1 in the *FVII* promoter region, as we showed that HIF2 $\alpha$  binding is not influenced by HDAC4 knockdown. It is also improbable that the synergistic activation of *FVII* is due to enhanced HIF2 $\alpha$  binding, as the ChIP assay revealed that HIF2 $\alpha$  binding under hypoxia is not enhanced by serum deprivation.

The tumor microenvironment is generally associated with hypoxia and poor nutrient supply due to inefficient vascularization (26). To inhibit apoptosis, cancer cells respond and adapt to these pressures by activating cellular mechanisms, such as angiogenesis, cell invasion, and signaling. Although the adaptive responses by HIFs are one of the major pathways induced under hypoxia, the co-operation of additional mechanisms and signaling by UPR assist cancer cell adaptation to hypoxia and nutrient deficiency, thereby promoting cell survival. In this study, we tested the hypothesis that cancer cells may sense serum deprivation and up-regulate transcription of those factors required for adaptation to hypoxia. The *FVII*-gene activation under real hypoxia in the presence of serum is rather moderate compared with that induced by CoCl<sub>2</sub> exposure; however, this may be due to the relatively weak expression levels of endogenous HIFs (9). It is also possible that side effects of CoCl<sub>2</sub> exposure including generation of reactive oxygen species and DNA damage (40) transmit signals to enhance Sp1–HIF2 $\alpha$ -dependent transcription. We found that activation of the *FVII* gene is synergistically enhanced in a deacetylation-dependent manner under the serum-free conditions, in which ER stress is induced. Given that HIF expression in OVSAYO cells is dramatically reduced under glucose-free conditions and weak under glucose-deprived conditions, it is plausible that the synergistic activation does not occur by glucose deprivation as opposed to serum starvation. Therefore, serum starvation-induced stress may provide an additional environmental cue to which cells adapt. We suggest that HDAC4 may be a stress-responsive deacetylase, such as sirtuins (12), when cells are exposed to hypoxia. We show that UPR is not responsible for this synergistic activation and further identification of the mechanisms responsible for this synergism is currently ongoing in our laboratory.

This study presents a novel transcriptional activation that is mediated by the interplay between HIF2 $\alpha$ /Sp1/HDAC4, enabling us to understand one of the mechanisms of thrombosis in CCC patients. Further exploration

of the genes and cancer phenotypes regulated by this HRE-independent mechanism will provide a greater understanding of cellular adaptation that manifests in response to environmental stresses, and may potentially lead to the development of new therapeutics for cancers targeting class IIa HDACs.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–7 and Supplementary Methods.

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