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DUSP22 Regulates Transcriptional Activity of HIF-1α

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Hypoxia is a state of deficiency of available oxygen in the blood and bodily tissues. Hypoxia is a common feature of cancer and almost all solid tumors contain hypoxic regions in which O_2 concentrations are greatly reduced compared to the surrounding normal tissue. The family of hypoxia-inducible factors (HIFs) that are master transcription regulators of the cellular response to hypoxia contains three α-subunits (HIF-1α, HIF-2α, and HIF-3α) and three β-subunits (HIF-1β, ARNT2 and ARNT3), respectively, which may give rise to different HIF heterodimers.[1,2] Hypoxia in growing tumors leads to stabilization of HIF-1α and induces the expression of several hypoxia responsive genes such as vascular endothelial growth factor (VEGF).[3] Hypoxic regions within the tumor can increase HIF-1α stability, transcriptional activity, and ultimately angiogenesis.

Under normoxic conditions, HIF-1α subunits of HIF have a very short half-life.[4] Under hypoxic condition, HIF-1α becomes stabilized and translocates into nucleus, where it dimerizes with HIF-1β, and the HIF-1 complex formed becomes transcriptionally active.[5,6] Activated HIF-1 complex then binds to hypoxia responsive element (HRE) in the promoter regions of target genes and associated with the transcriptional coactivators to induce gene expression.[7] The stability and subsequent transactivational function of HIF-1α is controlled by its posttranslational modification, such as hydroxylation, ubiquitination, acetylation, and phosphorylation.[8] Direct phosphorylation of HIF-1α by the mitogen-activated protein kinase (MAPK) pathway appears to occur after stabilization of the protein under normoxic or hypoxic conditions.[9-11] Phosphorylation does not have an effect on stability or on DNA binding of HIF-1α but increases the transcriptional activity of HIF-1.[10,12] One mechanism to explain increased transcriptional activity suggests that HIF-1β binds preferentially to phosphorylated form of HIF-1α.[13] However, the exact position of the phosphorylation sites as well as their effect on HIF-1α activity is not completely understood.

The recent advances revealed that HIF-1α is important for cancer therapy as well as drug identification. Therefore, we screened for protein tyrosine phosphatases (PTPs) that regulate transcriptional activity of HIF-1α using luciferase reporter assay. HIF-1α (residues 530-826) fused with the DNA binding domain of yeast GAL4 and a reporter plasmid (pFR-Luc) carrying 5 X GAL4 binding sequences in the promoter region that controls expression of the luciferase gene were used. Together with HIF-1α (530-826) expression plasmid, the pFR-Luc reporter plasmid and PTP expression plasmids were co-transfected into HepG2 cells. To mimic hypoxic condition, transfected cells were then treated with 150 μM $CoCl_2$. While overexpression of HIF-1α was sufficient for stimulation of the reporter gene, co-expression of PTPs affected HIF-1α transactivation activity. Transfection of PTPRT and PTP4A3 expression plasmids increased HIF-1α-mediated transactivation activity (more than 2-fold increase) (Table 1). In contrast, expression of dual-specificity phosphatase 22 (DUSP22) resulted in a 2-fold decrease in luciferase activity (Table 1).

To screen the intracellular interaction between endogenous HIF-1α and FLAG-PTPs, *in vivo* binding assays were carried out. HepG2 cells were transfected with FLAG-PTP expression plasmids. FLAG-PTPs were pulled down with *anti*-FLAG affinity agarose, followed by Western blotting with an *anti*-HIF-1α antibody. One of the strong interacting proteins was DUSP22 (data not shown).

To investigate that DUSP22 regulates HIF-1α transactivation activity, we performed HRE-driven luciferase reporter gene assays using HIF-1α, DUSP22 wild-type (WT) or catalytically inactive mutant DUSP22 C88S. To mimic hypoxic condition, transfected cells were treated with 150 μM $CoCl_2$.

Table 1. Luciferase assay-based screening of PTPs for HIF-1α transactivation activity. HepG2 cells were transiently co-transfected with reporter plasmid pFR-Luc and GAL4-HIF-1α (amino acids 530-826) fusion constructs, and gWIZ-GFP together with the pcDNA3.1-FLAG-PTPs. Cells were treated with 150 μM $CoCl_2$ for 6 h. Luciferase activities were normalized to GFP activities. The data are expressed as relative fold increase of luciferase units (RLU). All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test ($P < 0.001$). up = significant increase as compared to control; down = significant decrease as compared to control; — = no significant effect from control

PTP	Transcriptional Activity	PTP	Transcriptional Activity
PTPRS	-	PRL-1	-
PTPRH	-	PTP4A3	up
PTPRT	up	DUSP3	-
PTPRN	-	DUSP4	-
PTPN5	-	DUSP11	-
Cdc25C	-	DUSP12	-
ACP1	-	DUSP15	-
Laforin	-	DUSP22	down

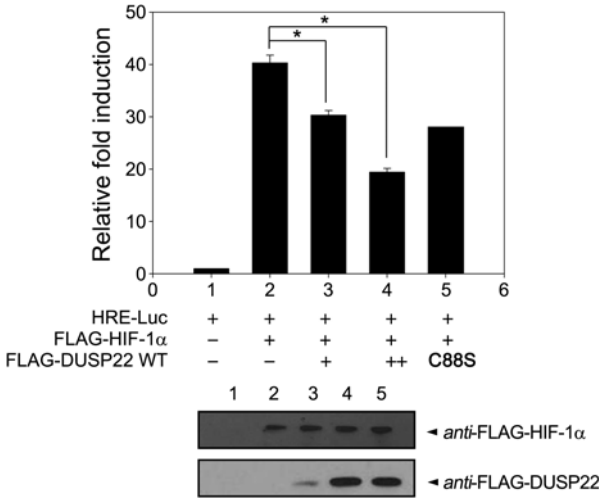

Figure 1. Repression of transcriptional activity of HIF-1α by DUSP22. HepG2 cells were transiently co-transfected with HRE-Luciferase reporter gene construct, HIF-1α expression plasmid and either increasing amounts of the pcDNA3.1-FLAG-DUSP22 (WT, 0.25, 0.5 μg) or pcDNA3.1-FLAG-DUSP22 (C88S, 0.5 μg). At 24 h after transfection, cells were exposed to 150 μM $CoCl_2$ for 6 h. Luciferase activities were normalized to GFP activities. HIF-1α transcriptional activity was measured by RLU. The protein expression levels were assessed by immunoblotting using *anti*-FLAG antibodies. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*$P < 0.001$).

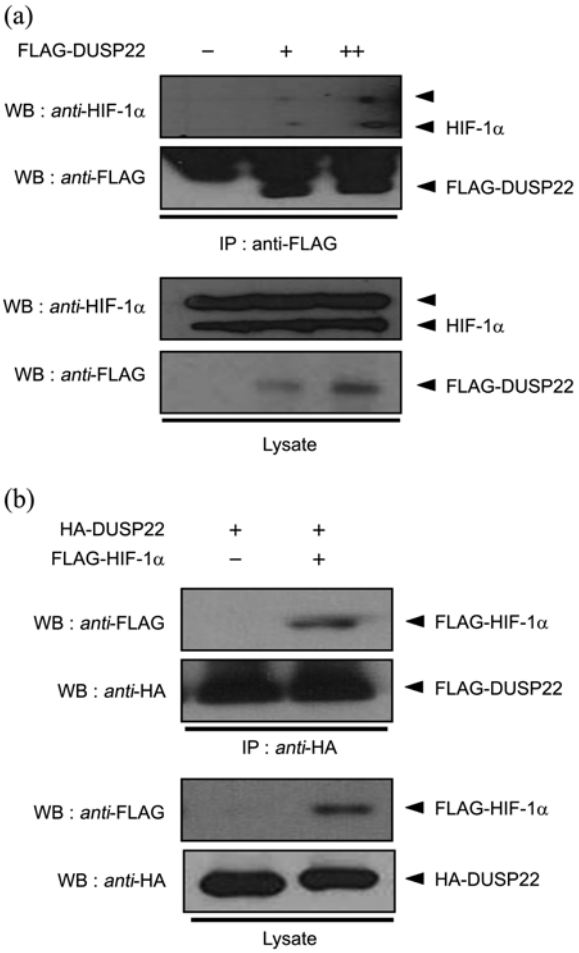

Figure 2. Interaction between HIF-1α and DUSP22. (a) HepG2 cells were transfected with FLAG-DUSP22 using 0.5 μg (+) or 1 μg (++). After whole cell lysates were immunoprecipitated with *anti*-FLAG M2-agarose, Western blotting analysis was performed using indicated antibodies. (b) HepG2 cells were co-transfected using HA-DUSP22 with or without FLAG-HIF-1α. After 30 h of transfection, cells were lysed and immunoprecipitated with *anti*-HA antibody. Immunoprecipitates were subjected to Western blotting with an *anti*-FLAG antibody.

As shown in Figure 1, DUSP22 suppressed HIF-1α transactivation activity in a dose-dependent manner. However, the catalytically inactive DUSP22 C88S mutant did not lead to strong inhibition of HIF-1α. Thus, these results suggest that DUSP22 regulates HIF-1α transactivation activity and its phosphatase activity is necessary for regulation.

In order to confirm the interaction of DUSP22 with endogenous HIF-1α *in vivo*, we examined the coprecipitation of endogenous HIF-1α and FLAG-DUSP22 from transiently transfected HepG2 cells. Cell lysates were incubated with *anti*-FLAG affinity agarose, followed by Western blot experiment (Fig. 2(a)). Consistent with the interaction in binding assay-based screening, the endogenous HIF-1α was co-immunoprecipitated with FLAG-DUSP22. Likewise, HepG2 cells co-transfected with FLAG-tagged HIF-1α and HA-tagged DUSP22 expression plasmids showed the pattern of co-immunoprecipitated HIF-1α and DUSP22 (Fig. 2(b)). Taken together, these results indicate that DUSP22 interacts with HIF-1α.

In this study, we have shown the evidence that DUSP22 regulates HIF-1α-dependent transcriptional activation under hypoxic conditions and DUSP22 interacts with HIF-1α. Sodium orthovanadate, an inhibitor of tyrosine phosphatases, increased not only the basal level of HIF-1 activity but also HIF-1α protein levels in hypoxic cells, suggesting that protein phosphatases are involved in the regulation of HIF-1α in several ways.[14] Direct phosphorylation of HIF-1α by ERK and p38 leading to increased HIF-1α transactivation has been reported by several groups.[10,11,15-17] It is simply possible that DUSP22 represses HIF-1α transcriptional activity *via* dephosphorylation of HIF-1α. The detailed regulatory mechanism needs to be elucidated.

Solid tumors frequently display severe hypoxia in their central regions. This is the major significance in clinical studies and treatment strategies since severe hypoxic areas are more resistant to chemotherapy and radiation. Therefore, a detailed understanding of the signaling pathways involved in HIF-1α regulation is essential for generating new therapeutic target for tumor treatment. The results obtained in this work reveal DUSP22 might be a potential new target to modulate angiogenesis in diseases such as cancer or ischemic cardiovascular disease.

Experimental Section

Cell Culture and Transfection. HepG2 (human hepatoma cell line) cells were maintained at 37 °C in DMEM (Invitro-

gen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin in the presence of 5% CO_2. For transient transfection, 1.4×10^6 cells were plated in each 60-mm cell culture plate, grown overnight, and transfected with DNA using Lipofectamine (Invitrogen).

Purification of 6 x His-tagged Proteins. Purification of 6 x His-tagged proteins was carried out as previously described.[18]

Luciferase Assay. HepG2 cells were cultured in 60-mm dishes and transfected with 0.5 μg each of the pFR-Luc, pFA-CMV-HIF-1α (amino acid 530-826) and gWIZ-GFP with or without pcDNA3.1-FLAG-PTPs (0.5 or 1 μg) using Lipofectamine (Gibco, Grand Island, NY, USA). For HRE-reporter assay, HepG2 cells transfected by Lipofectamine with HRE-luc reporter plasmid, pCMV-3 X FLAG 7.1-HIF-1α, and either increasing amounts of the pcDNA3.1-FLAG-DUSP22 (WT) or pcDNA3.1-FLAG-DUSP22 (C88S). Total amounts of DNA were equalized with empty plasmids. After 5 h of transfection in serum free medium, an equal volume of medium containing 10% FBS was added, and the cells were incubated for an additional 24 h. The medium was then replaced with DMEM in the presence of 150 μM $CoCl_2$ for 6 h. Cells were lysed in reporter lysis buffer (Promega, Madison, WI, U.S.A.). Cell extracts were analyzed with the luciferase reporter assay system using a Lumat LB 9501 Berthold Luminometer. The relative fold induction of luciferase activity was determined and normalized to GFP activity. All luciferase assays were repeated at least three times.

***In vivo* Binding Assays and Western Blotting Analysis.** HepG2 cells were transfected with PTPs expression plasmids, further incubated for 30 h, and lysed in PTP lysis buffer (0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, and 1 mM phenylmethanesulfonyl fluoride 1 ug/mL aprotinin) for 20 min at 4 °C, followed by centrifugation at 13,000 rpm for 20 min. For immunoprecipitation assays, the supernatants were pre-cleaned with 20 μL of protein A/G–agarose bead (50% slurry) and then incubated at 4 °C overnight with 20 μL of fresh protein A/G bead in the presence of appropriate antibodies. For PTPs binding screening, the soluble fractions were incubated with 20 μL of *anti*-FLAG M2 agarose (Sigma, Bucks, Switzerland) at 4 °C for overnight with rotation. After binding, the beads were washed three times in the phosphate buffered saline (PBS), resuspended in SDS sample buffer, and boiled for 10 min. Samples were analyzed by Western blotting using appropriate antibodies to detect protein expression. Antibodies against FLAG were purchased from Sigma. Antibody against HIF-1α was purchased from Abcam (Cambridge, MA, USA). The protein bands were visualized by the ECL detection system (PIERCE, Rockford, IL, USA)

Statistical Analysis. Statistical analysis of variances between two different experimental groups was performed with Tukey's post hoc comparison test using SPSS (Version K21). All experiments were repeated at least three times. The levels are considered significant for $p < 0.001$ (shown as *asterisk*).

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