Biochemical and Biophysical Research Communications 444 (2014) 36–43

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential roles of Sirt1 in HIF-1α and HIF-2α mediated hypoxic responses

Haejin Yoon, Seung-Hyun Shin, Dong Hoon Shin, Yang-Sook Chun, Jong-Wan Park *

Departments of Biomedical Sciences and Pharmacology, Ischemic/Hypoxic Disease Institute, Cancer Research Institute, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, Republic of Korea

ARTICLE INFO

Article history:
Received 20 December 2013
Available online 11 January 2014

Keywords:
Sirtuin 1
Lysine acetylation
Hypoxia
Hypoxia-inducible factors
Transcriptional activity

ABSTRACT

Hypoxia-inducible factors 1α and 2α (HIF-1α and HIF-2α) determine cancer cell fate under hypoxia. Despite the similarities of their structures, HIF-1α and HIF-2α have distinct roles in cancer growth under hypoxia, that is, HIF-1α induces growth arrest whereas HIF-2α promotes cell growth. Recently, sirtuin 1 (Sirt1) was reported to fine-tune cellular responses to hypoxia by deacetylating HIF-1α and HIF-2α. Yet, the roles of Sirt1 in HIF-1α and HIF-2α functions have been controversial. We here investigated the precise roles of Sirt1 in HIF-1α and HIF-2α regulations. Immunological analyses revealed that HIF-1α K674 and HIF-2α K741 are acetylated by PCAF and CBP, respectively, but are deacetylated commonly by Sirt1. In the Gal4 reporter systems, Sirt1 was found to repress HIF-1α activity constantly in ten cancer cell-lines but to regulate HIF-2α activity cell type-dependently. Moreover, Sirt1 determined cell growth under hypoxia depending on HIF-1α and HIF-2α. Under hypoxia, Sirt1 promoted cell proliferation of HepG2, in which Sirt1 differentially regulates HIF-1α and HIF-2α. In contrast, such an effect of Sirt1 was not shown in HCT116, in which Sirt1 inactivates both HIF-1α and HIF-2α because conflicting actions of HIF-1α and HIF-2α on cell growth may be offset. Our results provide a better understanding of the roles of Sirt1 in HIF-mediated hypoxic responses and also a basic concept for developing anticancer strategy targeting Sirt1.

1. Introduction

Most solid tumors contain hypoxic areas where oxygen is poorly delivered. Even cancer cells cannot survive under severe hypoxia, a part of cell population surrounding hypoxic areas can cope with hypoxia and forms hypoxia-resistant clones, which become more malignant than parent cancer by acquiring phenotypes of angiogenesis, invasion, and drug-resistance [1,2]. Hypoxia-inducible factor 1 and 2 (HIF-1 and HIF-2) play central roles in governing cancer cell fate under hypoxia [3,4]. HIF-1 or HIF-2 is the heterodimeric transcription factor consisting of HIF-1α and HIF-1β or HIF-2α and HIF-1β, respectively. The α-subunits act as the prime transcription factors and the β-subunit (alternatively named ARNT) helps α-subunits bind to DNA [5]. Despite the structural similarities of HIF-αs, many lines of evidence suggest that they have distinct roles in determining cancer cell fate [6]. For instance, HIF-1α sand HIF-2α target different gene sets, that is, the former induces genes for glycolysis and apoptosis but the latter does for cell survival and differentiation [7]. In addition, they regulate cell growth in opposite ways. HIF-1α inhibits cell proliferation, which is attributed to the activation of p53 tumor suppressor and the inhibition of proto-oncogenes c-Myc and β-catenin [8–10]. In contrast, HIF-2α promotes cell growth by inactivating p53 and by activating c-Myc and β-catenin [11–13]. According to the scenario on such the opposing roles of HIF-αs, we could manipulate cancer cell fate toward growth arrest by selectively controlling HIF-1α and HIF-2α. If so, this may be an ideal strategy to kill hypoxic cancer cells that survive HIF-dependently. To realize this strategy, it is needed to find the upstream regulating HIF-1α and HIF-2α. Given several literatures published recently [14,15], sirtuin 1 (Sirt1) could be the candidate that differentially regulates HIF-1α and HIF-2α.

Silent information regulator 2 (Sir2) was originally identified in yeast and drosophila, and characterized to mediate gene silencing and lifespan extension [16]. Of seven mammalian homologs (sirtuins, Sirt1–7) of Sir2, Sirt1 is considered as the prototype of sirtuin because it is most homologous to Sir2 [17]. Sirt1 functions to remove the acetyl moiety from acetylated lysine residues in histones and non-histone proteins. Sirt1 requires NAD^+ as a cofactor, which is common in the class III HDAC family. Functionally, Sirt1 induces heterochromatin by deacetylating $H_4K_{16}Ac$, H_3K_9Ac and $H_1K_{26}Ac$,

Abbreviations: Ac-K, acetylated lysine residue; CAD, C-terminal transactivation domain; CBP, CREB-binding protein; DBD, DNA-binding domain; HDAC, histone deacetylase; HIF, hypoxia-inducible factor; PCAF, p300/CBP-associated factor; Sirt1, sirtuin 1.

* Corresponding author. Fax. +82 2 745 7996.
E-mail address: parkjw@snu.ac.kr (J.-W. Park).

0006-291X/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2014.01.001

leading to gene silencing [18]. In addition, Sirt1 participates in diverse processes, such as differentiation, inflammation, energy metabolism, and myogenesis by targeting transcription factors, such as, p53, FOXO, E2F1, NF-κB, PGC-1α, LXR, and MyoD [19]. Notably, Sirt1 has been reported to regulate hypoxic response by directly deacetylating HIF-1α and HIF-2α. By mass analysis, HIF-1α was identified to be acetylated at Lys674 by p300/CBP-associated factor (PCAF) and to be deacetylated by Sirt1 [15]. HIF-2α was demonstrated to be acetylated at lysines 385, 685 and 741 by CREB-binding protein (CBP) but to be reversed by Sirt1 [14,20]. As a consequence of the Sirt1-mediated deacetylation, HIF-1α is functionally inhibited due to p300 dissociation from the C-terminal transactivation domain (CAD) of HIF-1α [15]. Reversely, the HIF-2-driven gene expression is augmented in hepatoma cells by Sirt1 and down-regulated in the liver and the kidney of Sirt1-deficient mice [14]. Accordingly, Sirt1 seems to be a potential target for selectively controlling HIF-1α and HIF-2α. However, the roles of Sirt1 in the HIF signaling pathways are still controversial because a few studies reported conflicting results [14,21].

We designed this study to understand the precise roles of Sirt1 in the HIF-1α and HIF-2α signaling pathways. The Sirt1-mediated deacetylation of HIF-1α and HIF-2α were detected with specific antibodies recognizing Sirt1-targeted acetylated lysine residues, and the specific roles of Sirt1 in HIF-1α and HIF-2α functions were examined using the Gal4-luciferase reporter system. Moreover, how Sirt1 determines cancer cell fate under hypoxia was investigated by checking cell growth and gene expression profile.

2. Materials and methods

2.1. Reagents and antibodies

Culture media and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA) and Sigma–Aldrich (St. Louis, MO), respectively. Anti-HIF-1α antibody was raised from rabbits against human HIF-1α [22]. Acetylated-lysine antibody was from Cell Signaling (Danvers, MA). Sirt1, HIF-2α, α-tubulin, Gal4(DBD), and CBP primary antibodies and HRP-conjugated secondary antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA); anti-HA-tag and anti-Flag-tag antibody from Sigma–Aldrich.

2.2. Cell lines and culture conditions

HEK293T (human embryonic kidney), HepG2 (hepatoma), Hep3B (hepatoma), HCT116 (colorectal cancer), CT26 (colorectal cancer), AGS (gastric cancer), HeLa (cervical cancer), H1299 (lung cancer), HT-1080 (fibrosarcoma), U87MG (glioblastoma) and PNT2 (human prostate epithelial) cell-lines were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured at 5% CO_2 in Eagle's medium, RPMI-1640 or MEM, which were supplemented with 10% heat-inactivated FBS. Gas tension in an incubator was 20% O_2/5% CO_2 for normoxia or 1% O_2/5% CO_2 for hypoxia.

2.3. Antibody generation

Antibodies against acetylated K674 of HIF-1α and acetylated K741 of HIF-2α were raised from rabbits (New Zealand white). Rabbits were immunized with Keyhole limpet haemocyanin (KLH)-conjugated synthetic peptides, which contain Sirt1-tartgetted acetylated lysine residues (acetylated K674 of HIF-1α and acetylated K741 of HIF-2α), and specific antibodies were purified by affinity chromatography using the lysyl-acetylated peptides. The structures of synthetic peptides used as antigens were illustrated in Fig. 1A.

2.4. Preparation of plasmids, siRNAs and transfection

The HA-tagged HIF-1α and Myc/His-Sirt1 plasmids were kindly given by Dr. Eric Huang (University of Utah) and Dr. Junjie Chen (University of Texas), respectively. The Flag-tagged HIF-2α and PCAF plasmids were constructed by RT-PCR and the CBP plasmid was donated by Addgene (Cambrige, MA). The sequences of Sirt1-targeting siRNAs used were 5′-UUCAACAUUCCUAGAAGUUU-GUACUUC-3′ (si-Sirt1 #1) and 5′-ACAGUUUCAUAGAGCCAUGAA-GUAU-3′ (si-Sirt1 #2), and the non-targeting siRNA sequence was 5′-AUGAACGUGAAUUGCUCAA-3′. For overexpression or knock-down, cells at 40% density were transfected with plasmids (1 μg per 100-mm dish) or siRNAs (40 nM concentration) using calcium phosphate for HEK293T cells or using Lipofectamine (Invitrogen) for cancer cells. The transfected cells were allowed to be stabilized for 48 h before experiments.

2.5. Gal4-reporter plasmids and luciferase assay

To evaluate HIF-1α CAD (aa. 597–826) and HIF-2α CAD (675–870) activities, the CADs were cloned using the *pfu* DNA polymerase-based PCR of human HIF-1α and HIF-2α cDNAs, and inserted into the 3′-end of the Gal4 DBD (DNA-binding domain) gene in the plasmid CMX-Gal4, which was given by Dr. Eric Huang (University of Utha), using blunt-end ligation. Cells were co-transfected with the Gal4-CAD fusion plasmids and the Gal4-promoter/luciferase reporter plasmid using calcium phosphate or Lipofectamine. The CMV/β-galactosidase plasmid was also co-transfected with the reporters to verify transfection efficiency. Final DNA and siRNA doses were adjusted by adding pcDNA and non-targeting siRNA, respectively. After being stabilized for 48 h, transfected cells were incubated under normoxic or hypoxic conditions for 16 h, and then lysed to determine luciferase and β-galactosidase activities. The luciferase activity of each sample was divided by the galactosidase activity in the same sample and presented as a relative value to the normoxic control.

2.6. Immunoblotting and immunoprecipitation

Cell lysates were separated on SDS–polyacrylamide gels, and transferred to Immobilon-P membranes (Millipore; Bedford, MA). Membranes were blocked with 5% skim milk for 1 h and then incubated overnight at 4 °C with a primary antibody diluted 1:1000 in the blocking solution. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h, and stained using the ECL kit (Thermo; Rockford, IL). To analyze protein interactions, cell lysates were incubated with anti-HA, anti-Flag or anti-Gal4 antibody for 4 h at 4 °C, and the immune complexes were pulled down by protein A/G beads (Santa Cruz, CA). The bound proteins were eluted in a denaturing SDS sample buffer, loaded on SDS–PAGE, and immunoblotted.

2.7. Quantitative RT-PCR

RNAs were isolated using TRIZOL (Invitrogen), and reverse-transcribed using Quantitect kit (Qiagen). Real-time PCR was performed in triplicates with SYBR® Green PCR Master Mix (Applied Biosystems) and detected by CFX ConnectTM System (Bio-Rad). PCR conditions were 40 cycles of 94 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s. Data were analyzed using CFX Manager Software (Bio-Rad). Values were normalized to β-actin levels. Primer sequences (5′ to 3′) for PDK1 (pyruvate dehydrogenase kinase 1), BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3), CITED2 (CBP/p300-interacting transactivator with Glu/Asp-rich domain 2), cyclin D1, and β-actin were CCTTTGAGGAAAATTGACAG and AACCCTCTAGGGAATACAGC; TTCTGAAAGTTTTCCTTCCA and

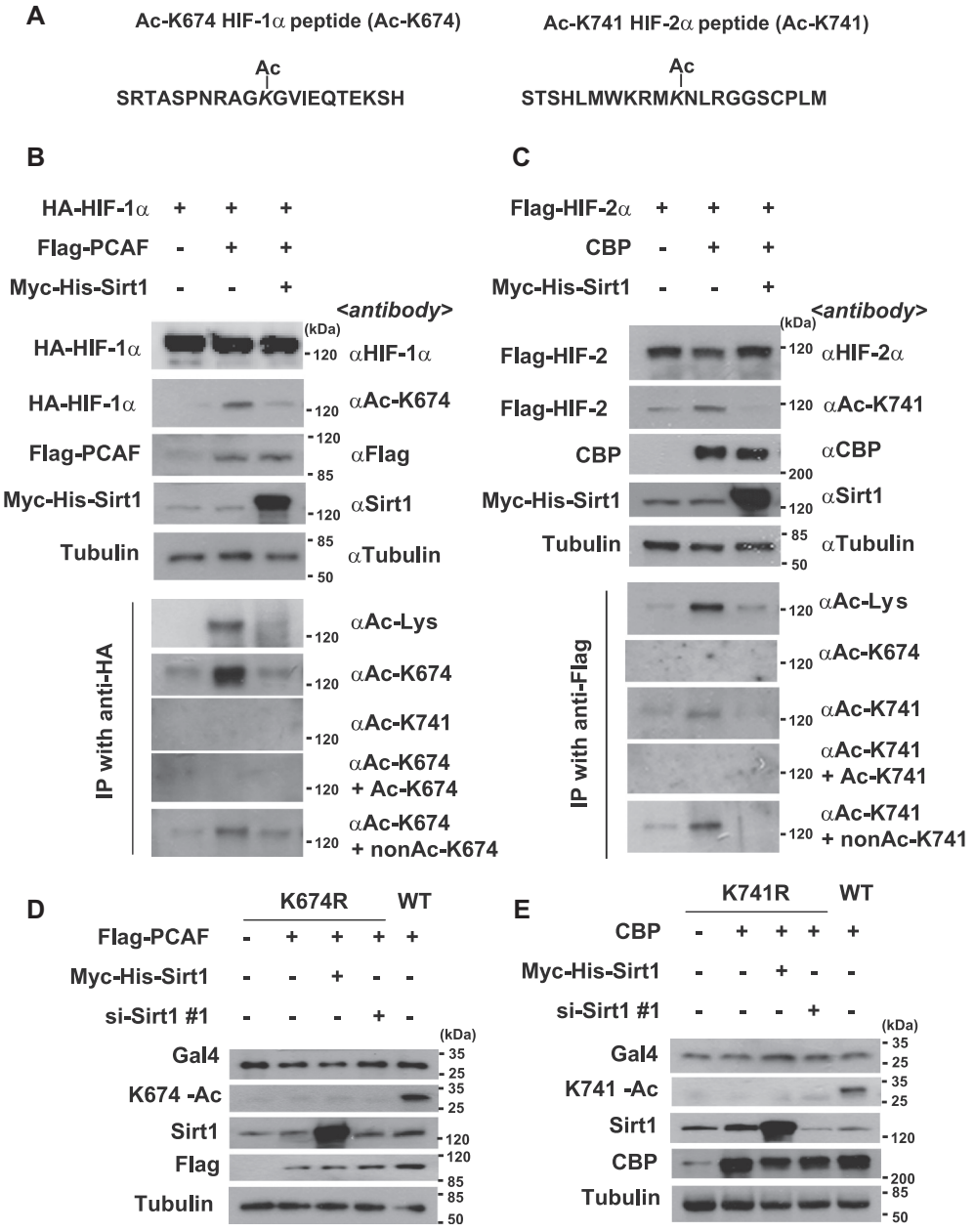

Fig. 1. Sirt1 deacetylates HIF-1α at Lys674 and HIF-2α at Lys741. (A) The structures of lysyl-acetylated HIF-1α and HIF-2α peptides used as antigens to generate antibodies against HIF-1α acetylated at K674 and HIF-2α acetylated at K741. (B) HEK293T cells were transfected with HA-HIF-1α, Flag-PCAF, or/and Myc-His-Sirt1 plasmids. After being stabilized for 24 h, cells were incubated under hypoxic conditions for 8 h. Proteins in cell lysates were precipitated with anti-HA affinity beads for 4 h. Whole cell lysates and immunoprecipitated proteins were electrophoresed and immunoblotted with the indicated antibodies. To verify the specificity of the antibody against acetylated K674, acetylated or non-acetylated K674 peptide was preincubated with the antibody 1 h prior to immunoblotting. (C) HEK293T cells were transfected with Flag-HIF-2α, CBP, or/and Myc-His-Sirt1 plasmids. After 8 h-hypoxia, cells were lysed and precipitated with anti-Flag affinity beads. Whole cell lysates and immuno precipitated proteins were subjected to immunoblotting with the indicated antibodies. Acetylated or non-acetylated K741 peptide was used to verify the antibody. (D) Gal4-HIF-1α or its K674R mutant plasmid was co-transfected with Flag-PCAF, Myc-His-Sirt1, or Sirt1 siRNA (si-Sirt1) into HEK293T cells. After cells were incubated under hypoxia for 8 h, the HIF-1α acetylation at K674 was checked by immunoblotting. (E) The acetylation of Gal4-HIF-2α and its K741R mutant under hypoxia were checked by immunoblotting.

TGTTGCAAGCTCAGAAGTAA; ACCCACCTCCCTTATGTAGT and CCAA CTAATGCAATTTTTCC; GGAGGAGAACAAACAGATCA and GTAGGA CAGGAAGTTGTTGG; ATCCACGAAACTACCTTCAA and TCTTGATCTT CATTGTGCTG, respectively.

2.8. Cell population and cell cycle analyses

Cell population was evaluated using the MTT labeling kit (Sigma–Aldrich). Cells in 100 μl of medium were grown in 96-well plates and were incubated with 100 μl of the MTT reagent (5 mg/ml) in the CO_2 chamber for 3 h. After solubilizing the blue formazan crystal with acidified isopropanol, we determined the formazan levels at 570 nm. For analyzing cell cycle, cells were cultured at 30–40% confluence. After normoxic or hypoxic incubation for 24 h, cells were harvested and fixed in 75% ethanol for 2 h. Cells were labeled with propidium iodide (0.05 mg/ml) with RNase A (0.5 mg/ml) in the dark for 30 min. DNA contents were analyzed by cytofluorometry (BD-FACS Canto II: BD Bioscience). Propidium iodide was excited at 488 nm and detected at 650 nm.

2.9. Statistics

All data were analyzed using Microsoft Excel 2007 software, and results are expressed as means and standard deviations. We

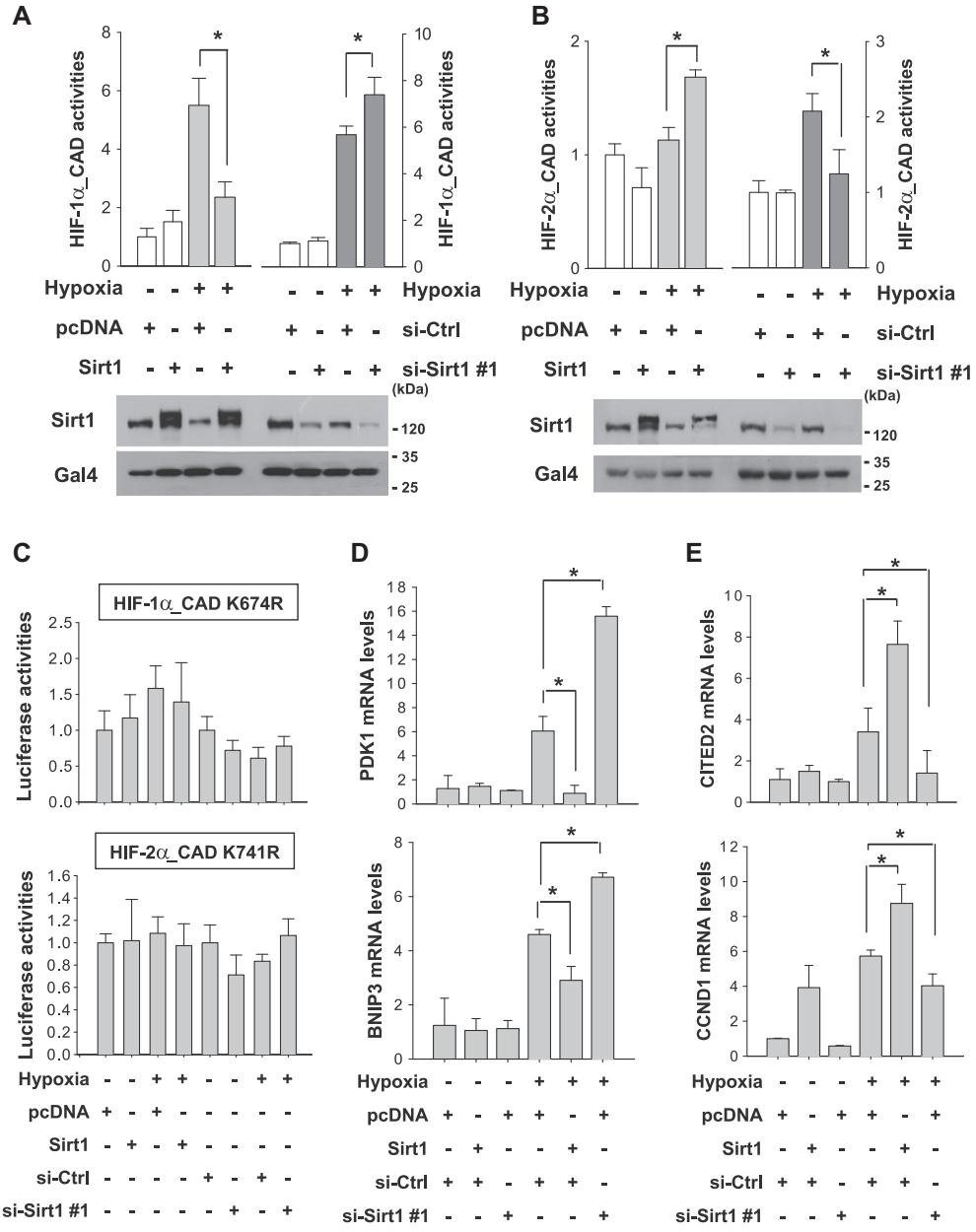

Fig. 2. Sirt1 inactivates HIF-1α CAD but activates HIF-2α CAD in HEK293T cells. HEK293T cells, in which Sirt1 plasmid or siRNA-Sirt1 was co-transfected with Gal4-luc reporter and Gal4-HIF-1α-CAD (A) or Gal4-HIF-2α-CAD (B), were incubated under normoxic or hypoxic conditions for 24 h. Luciferase activities were measured by a luminometer and presented as relative values (means and SDs, $n = 4$) to the normoxic, pcDNA control value. Cellular levels of Sirt1 and Gal4-CADs were analyzed by Western blotting. (C) HEK293T cells, which had been cotransfected with Gal4-HIF-1α-CAD K674R or Gal4-HIF-2α CAD K741R and other plasmids indicated, were incubated under normoxic or hypoxic conditions for 24 h. Luciferase activities were presented as relative values (means and SDs, $n = 4$) to the normoxic pcDNA control. (D and E) HEK293T cells, which had been transfected as indicated, were incubated under normoxic or hypoxic conditions for 24 h. The mRNA levels of HIF-1α (D) or HIF-2α (E) downstream genes were reverse-transcribed and quantified by real-time PCR. All experiments were performed three times and results are expressed as means and SDs. * denotes $P < 0.05$ versus the hypoxic control value.

used the unpaired, two-sided Student *t*-test to compare reporter activities and cell populations. Statistical significances were considered when *P* values were less than 0.05.

3. Results and discussion

3.1. Sirt1 deacetylates HIF-1α and HIF-2α at lysine residues in their transactivation domains

Previous mass analyses revealed that HIF-1α at K674 and HIF-2α at K741 are acetylated by PCAF and CBP acetyl-transferases, respectively. Moreover, the levels of lysyl-acetylated HIF-1/2α proteins were shown to be reduced by Sirt1 overexpression, but the acetylation was detected using the antibody recognizing acetylated lysine non-selectively [14,15,20]. To confirm that the lysine residues are specific targets of Sirt1, we developed the antibodies against acetylated K674 of HIF-1α and against acetylated K741 of HIF-2α using synthetic peptides (Fig. 1A). HEK293T cells were cotransfected with HA-HIF-1α, PCAF, and/or Sirt1 plasmids and incubated for 2 days. In both total cell lysates and HIF-1α-enriched immunoprecipitates, anti-Ac-K674 antibody could detect the acetylation of HIF-1α at K674. The lysyl-acetylation of HIF-1α was induced by PCAF, which was reversed by Sirt1 (Fig. 1B). Likely, we analyzed the HIF-2α acetylation using anti-Ac-K741 antibody and

confirmed that the K741 residue of HIF-2α is acetylated and deacetylated by CBP and Sirt1, respectively (Fig. 1C). When the antibodies were pre-incubated with K674 or K741 peptides, the immunoreactions were almost completely blocked by the acetylated peptides, but were not by the non-acetylated peptides. These results indicate that the antibodies specifically react with K674-acetylated HIF-1α or K741-acetylated HIF-2α. To further check the specificities of developed antibodies, we expressed HIF-1/2α peptides lacking the specific lysines targeted by Sirt1. Anti-Ac-K674 and anti-Ac-K741 antibodies did not recognize the mutated peptides, which further supports the specificities of the antibodies (Fig. 1D and E). These results make sure that Sirt1 deacetylates HIF-1α at K674 and HIF-2α at K741. Moreover, the antibodies recognizing the Sirt1 target sites will be materials useful to search for new chemicals targeting the Sirt1-HIF-1/2 pathways.

3.2. Sirt1 differentially regulates the transcriptional activities of HIF-1α and HIF-2α

How Sirt1 regulates HIF-1α and HIF-2α remains controversial so far. Given two literatures showing that Sirt1 functionally regulates HIF-1α and HIF-2α through their CADs [14,15], we designed the luciferase reporter systems to evaluate the functionalities of HIF-1α and HIF-2α CADs. The structures of Gal4-CAD fusion proteins and the principle of the reporter assays are illustrated in Supplemental Fig. 1. In HEK293T cells cotransfected with the reporter plasmids and Sirt1 plasmid or siRNA, expressed Sirt1 repressed the transcriptional activity of HIF-1α_CAD (Fig. 2A), but facilitated that of HIF-2α_CAD (Fig. 2B). Sirt1-silencing RNAs regulated the CADs in opposite ways. To rule out the off-target effect of the Sirt1 siRNA (si-Sirt1 #1) used, we designed another Sirt1 siRNA (designated si-Sirt1 #2) and found that the second

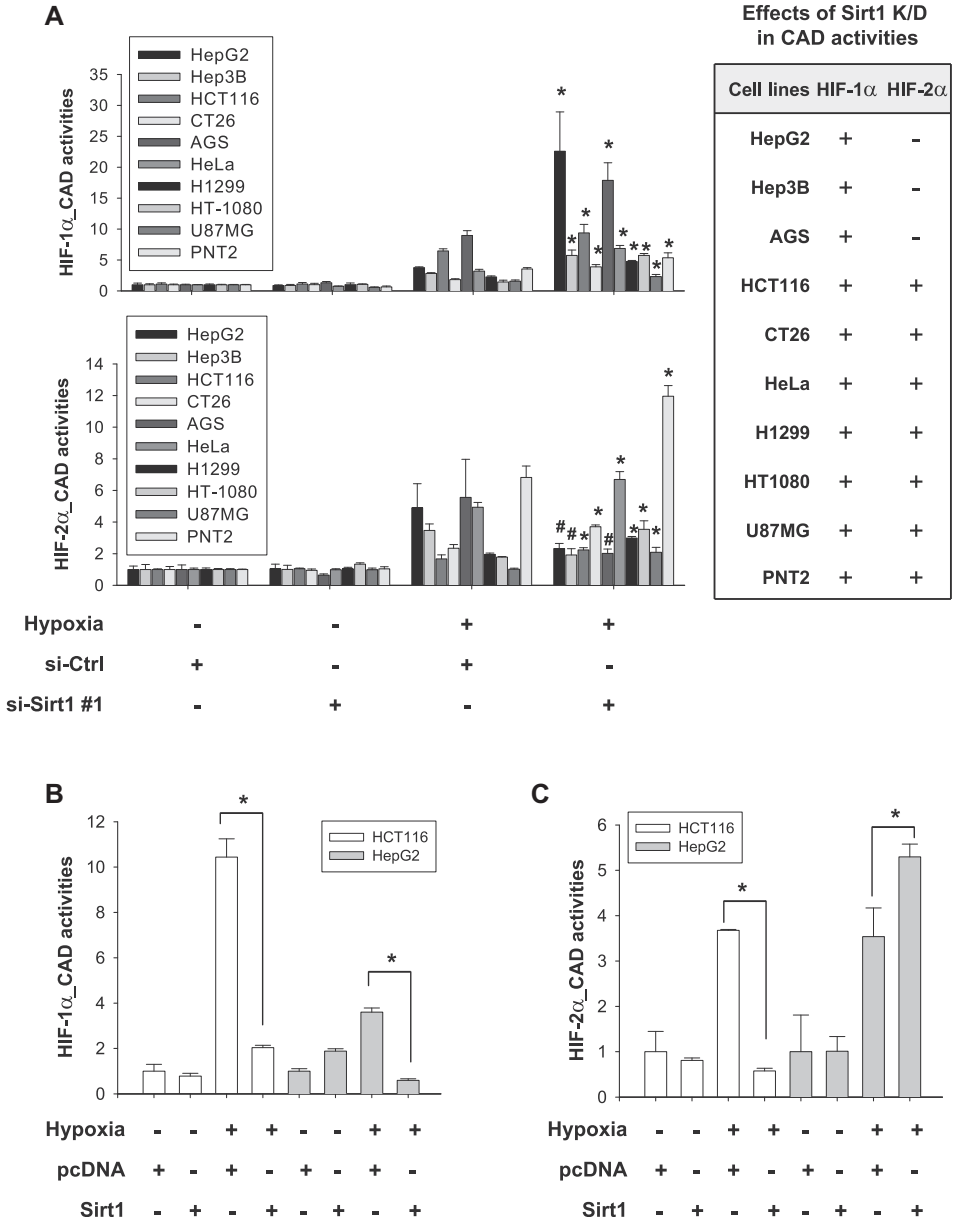

Fig. 3. Sirt1 inhibits HIF-1α constantly but regulates HIF-2α cell context-dependently. (A) Various cancer cells were co-transfected with the siRNAs and Gal4-HIF-1α-CAD or Gal4-HIF-2α-CAD plasmid, and were subjected to normoxia or hypoxia for 24 h. Results (means and SDs, $n = 4$) are presented as relative activities versus the normoxic si-control value. * and # present that the si-Sirt1 value is statistically ($P < 0.05$) higher and lower than the si-control value, respectively. The effects of Sirt1 knock-down on the hypoxic activation of HIF-αs at the cell lines are summarized in the right table. (B and C) HCT116 or HepG2 cells, which had been cotransfected with Gal4-HIF-1α-CAD (B) or Gal4-HIF-2α-CAD (C) and Sirt1 plasmids, were incubated under normoxic or hypoxic conditions for 24 h. Results (means and SDs, $n = 4$) are presented as relative luciferase activities versus the normoxic control activity.

one also regulates the activities of HIF-1α and HIF-2α CADs in opposite ways (Supplemental Fig. 2A). We next analyzed the transcriptional activities of mutated Gal4-CADs, namely, HIF1A_CAD K674R and HIF2A_CAD K741R. Consequently, the activities of both mutated CADs were not substantially regulated by Sirt1 under hypoxia (Fig. 2C and Supplemental Fig. 2B). We also checked the mRNA levels of PDK1 and BNIP3 (HIF-1α downstream genes) and those of CITED2 and CCND1 (HIF-2α downstream genes) in HEK293T cells. The hypoxic expressions of PDK1 and BNIP3 were attenuated by Sirt1 overexpression but augmented by two Sirt1 siRNAs (Fig. 2D and Supplemental Fig. 3A). In contrast, CITED2 and CCND1 levels were induced under hypoxia depending on Sirt1 expression (Fig. 2E and Supplemental Fig. 3B). When we examined whether Sirt1 regulates HIF-αs at their protein levels, HIF-1α and HIF-2α both were found to be expressed independently of Sirt1 (Supplemental Figs. 2C and 3C). These results strongly support our notion that Sirt1 differentially regulates the transcriptional activities of HIF-1α and HIF-2α under hypoxia at least in HEK293T cells.

3.3. Sirt1 inhibits HIF-1α CAD constantly but regulates HIF-2α CAD cell context-dependently

We next examined how Sirt1 regulates HIF-1α and HIF-2α in cell-lines other than HEK293T. Ten cancer cell-lines were transfected with the Gal4-CAD reporter plasmids and incubated under

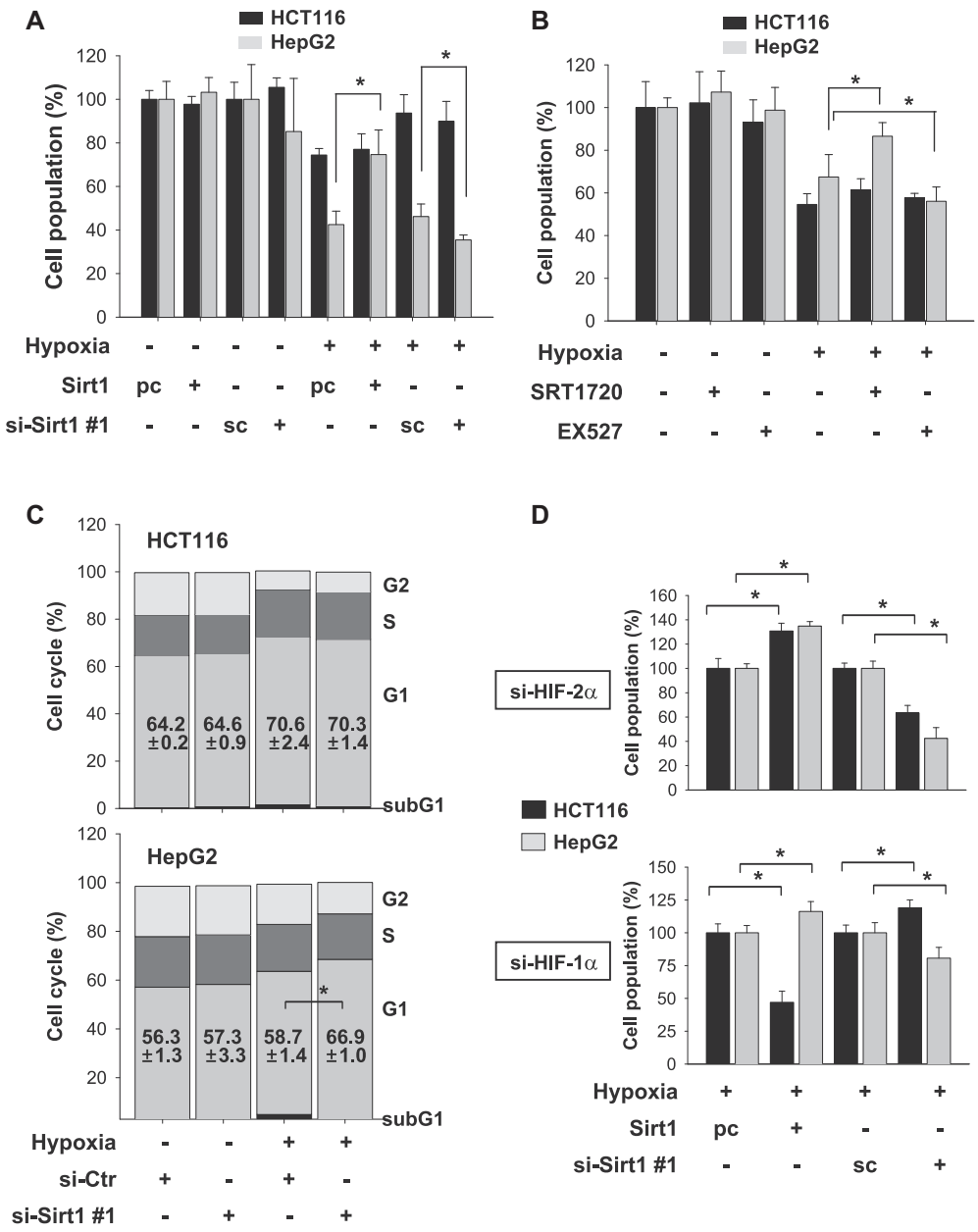

Fig. 4. Sirt1 determines cell growth under hypoxia by modulating HIF-1α and HIF-2α activities. (A) HCT116 or HepG2 cells transfected as indicated were incubated under normoxic or hypoxic conditions for 24 h, and cell population was analyzed by MTT-labeling method. Results (means and SDs, $n = 4$) are presented as relative values versus the normoxic control. pc: pcDNA, sc: control siRNA. (B) HCT116 or HepG2 cells transfected as indicated were treated with SRT1720 (100 nM) or EX527 (100 nM) 1 h prior to normoxic or hypoxic incubation for 24 h. Results (means and SDs, $n = 4$) are presented as relative MTT values versus the normoxic control. (C) After transfected HCT116 and HepG2 cells were incubated for 24 h as indicated, cell cycle was analyzed using propidium iodide. (D) HCT116 or HepG2 cells, which had been cotransfected with HIF-1α or HIF-2α siRNA and Sirt1 plasmid or siRNA, were incubated under hypoxia for 24 h, and cell population was analyzed by MTT-labeling method. * denotes $P < 0.05$ between two groups indicated in all panels.

normoxic or hypoxic conditions for 16 h. As was shown in HEK293T, HIF-1α CAD activity under hypoxia was significantly augmented by Sirt1 knock-down in all cell-lines tested, which further supports the Sirt1 inhibition of HIF-1α. Interestingly, HIF-2α CAD activity was inconstantly regulated by Sirt1 knock-down. Sirt1 knock-down inhibited HIF-2α CAD in three cell-lines, but activated that in seven cell-lines (Fig. 3A). To confirm the effects of Sirt1 on HIF-1/2α CAD activities, we co-transfected Sirt1 and Gal4-CAD reporter plasmids in HCT116 and HepG2 cells. HIF-1α CAD was repressed by Sirt1 overexpression in both cell-lines (Fig. 3B) whereas HIF-2α CAD showed differential responses to Sirt1 overexpression in the two cell-lines (Fig. 3C). Expressed Sirt1 inhibited HIF-2α CAD in HCT116, but stimulated that in HepG2, which further suggests that the effect of Sirt1 on HIF-2α CAD is variable cell context-dependently. Indeed, the mechanism underlying Sirt1-mediated HIF-2α activation was studied mainly in a hepatoma cell-line, Hep3B [14,20]. As was expected, two hepatoma cell-lines (Hep3B and HepG2) also showed such responses in the present study. In 70% of cancer cell-lines tested, however, Sirt1 was found to act as a negative regulator in the hypoxic activation of HIF-2α CAD. Some cell-specific factors may determine the functionality of HIF-2α CAD deacetylated by Sirt1.

3.4. Sirt1 promotes cell growth under hypoxia in HepG2 but not in HCT116

Many lines of evidence suggest that HIF-2α helps cells grow under hypoxia, but HIF-1α reversely acts [23]. Therefore, we checked the roles of Sirt1 in the hypoxic growths of HCT116 and HepG2 cells, which showed differential roles of Sirt1 in HIF-2α activity. After 24 h-hypoxia, the numbers of both cells was substantially reduced. Notably, HepG2 cell number under hypoxia was increased and decreased by Sirt1 overexpression and knock-down, respectively (Fig. 4A). Likewise, si-Sirt1 #2 reduced cell population under hypoxia in HepG2, but did not in HCT116 (Supplemental Fig. 4A). When HepG2 cells were treated with Sirt1 activity modulators under hypoxia, the cell number was increased and decreased by SRT1720 (Sirt1 activator) and Ex527 (Sirt1 inhibitor), respectively (Fig. 4B). In contrast, the hypoxic cell growth of HCT116 under hypoxia was independent of Sirt1 level and activity. In cell cycle analyses, the subG1 population (an apoptotic index) was not significantly affected in both cell lines by Sirt1 knock-down (Fig. 4C and Supplemental Fig. 5), suggesting that Sirt1 regulates cell proliferation, rather than cell death. Moreover, the G1 population in HepG2, not HCT116, under hypoxia was significantly increased by Sirt1 knock-down. Considering the Fig. 4A result showing that the number of hypoxic HepG2 cells was reduced by Sirt1 knock-down, Sirt1 knock-down is likely to induce cell-cycle arrest at the G1 phase in HepG2 cells under hypoxia. To examine the roles of HIF-1α and HIF-2α separately, one of HIF-αs were knocked-down in HCT116 and HepG2, and the knock-down efficiencies were verified using Western blotting (Supplemental Fig. 4B). When HIF-2α was suppressed (HIF-1α alone remained), Sirt1 showed positive effects in the cell growth of both cell-lines under hypoxia (Fig. 4D, top). Such an effect of Sirt1 may be attributed to the inactivation of HIF-1α, which is responsible for cell growth arrest during hypoxia. On the contrary, Sirt1 differentially regulated cell growth in HCT116 and HepG2 when HIF-1α was suppressed (HIF-2α alone remained). Sirt1 inhibited HCT116 cell growth but promoted HepG2 cell growth (Fig. 4D, bottom). The cell type-dependent actions of Sirt1 on cell growth may be attributable to the opposite effects of Sirt1 on HIF-2α activity in two cell-lines. These results also gave us a hint to explain why HCT116 cell growth was independent of Sirt1 level and activity (Fig. 4A and B). As Sirt1 inhibited both HIF-αs having conflicting actions on cell growth between each other, the effect of Sirt1 on cell growth may be offset in HCT116 cells.

3.5. Summary and conclusion

In the present study, we developed the specific antibodies recognizing lysyl-acetylated HIF-1/2α, and by using them demonstrated that HIF-1α K674 and HIF-2α K741 are acetylated by PCAF and CBP, respectively, but both are deacetylated by Sirt1. The Gal4-CAD reporter systems clearly revealed how Sirt1 regulates HIF-1α and HIF-2α, that is, Sirt1 represses HIF-1α activity in all cell-lines tested, but variably regulates HIF-2α activity cell type-dependently. HepG2 cells, in which Sirt1 regulates HIF-1α and HIF-2α in the opposite way, grew better under hypoxia when Sirt1 was up-regulated or activated. Our results provide a better understanding of the roles of Sirt1 in HIF-mediated hypoxic responses and also a basic concept that Sirt1 could be a potential target for treating malignant tumors exposed to hypoxia.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgments

This work was supported by a Grant (2010-0029948) funded by the National Research Foundation of Korea and by the Basic Science Research Program (2009-0090188) funded by the Ministry of Education, Science and Technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.001.

References

[1] D. Liao, R.S. Johnson, Hypoxia: a key regulator of angiogenesis in cancer, Cancer Metastasis Rev. 26 (2007) 281–290.
[2] J.M. Brown, W.R. Wilson, Exploiting tumour hypoxia in cancer treatment, Nat. Rev. Cancer 4 (2004) 437–447.
[3] G.L. Wang, B.H. Jiang, E.A. Rue, G.L. Semenza, Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension, Proc. Natl. Acad. Sci. USA 92 (1995) 5510–5514.
[4] J. Pouysségur, F. Dayan, N.M. Mazure, Hypoxia signalling in cancer and approaches to enforce tumour regression, Nature 441 (2006) 437–443.
[5] M.J. Calzada, L. del Peso, Hypoxia-inducible factors and cancer, Clin. Transl. Oncol. 9 (2007) 278–289.
[6] A. Loboda, A. Jozkowicz, J. Dulak, HIF-1 and HIF-2 transcription factors – similar but not identical, Mol. Cells 29 (2010) 435–442.
[7] C.J. Hu, L.Y. Wang, L.A. Chodosh, B. Keith, M.C. Simon, Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation, Mol. Cell. Biol. 23 (2003) 9361–9374.
[8] N. Goda, H.E. Ryan, B. Khadivi, W. McNulty, R.C. Rickert, R.S. Johnson, Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia, Mol. Cell. Biol. 23 (2003) 359–369.
[9] M. Koshiji, Y. Kageyama, E.A. Pete, I. Horikawa, J.C. Barrett, L.E. Huang, HIF-1alpha induces cell cycle arrest by functionally counteracting Myc, EMBO J. 23 (2004) 1949–1956.
[10] A. Kaidi, A.C. Williams, C. Paraskeva, Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia, Nat. Cell Biol. 9 (2007) 210–217.
[11] A.M. Roberts, I.R. Watson, A.J. Evans, D.A. Foster, M.S. Irwin, M. Ohh, Suppression of hypoxia-inducible factor 2alpha restores p53 activity via Hdm2 and reverses chemoresistance of renal carcinoma cells, Cancer Res. 69 (2009) 9056–9064.
[12] J.D. Gordan, J.A. Bertout, C.J. Hu, J.A. Diehl, M.C. Simon, HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity, Cancer Cell 11 (2007) 335–347.
[13] H. Choi, Y.S. Chun, T.Y. Kim, J.W. Park, HIF-2alpha enhances beta-catenin/TCF-driven transcription by interacting with beta-catenin, Cancer Res. 70 (2010) 10101–10111.

[14] E.M. Dioum, R. Chen, M.S. Alexander, Q. Zhang, R.T. Hogg, R.D. Gerard, J.A. Garcia, Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1, Science 324 (2009) 1289–1293.
[15] J.H. Lim, Y.M. Lee, Y.S. Chun, J. Chen, J.E. Kim, J.W. Park, Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha, Mol. Cell 38 (2010) 864–878.
[16] G. Blander, L. Guarente, The Sir2 family of protein deacetylases, Annu. Rev. Biochem. 73 (2004) 417–435.
[17] N. Dali-Youcef, M. Lagouge, S. Froelich, C. Koehl, K. Schoonjans, J. Auwerx, Sirtuins: the 'magnificent seven', function, metabolism and longevity, Ann. Med. 39 (2007) 335–345.
[18] P. Martínez-Redondo, A. Vaquero, The diversity of histone versus nonhistone sirtuin substrates, Genes Cancer 4 (2013) 148–163.
[19] S. Michan, D. Sinclair, Sirtuins in mammals: insights into their biological function, Biochem. J. 404 (2007) 1–13.
[20] R. Chen, M. Xu, R.T. Hogg, J. Li, B. Little, R.D. Gerard, J.A. Garcia, The acetylase/deacetylase couple CREB-binding protein/Sirtuin 1 controls hypoxia-inducible factor 2 signaling, J. Biol. Chem. 287 (2012) 30800–30811.
[21] R. Chen, E.M. Dioum, R.T. Hogg, R.D. Gerard, J.A. Garcia, Hypoxia increases sirtuin 1 expression in a hypoxia-inducible factor-dependent manner, J. Biol. Chem. 286 (2011) 13869–13878.
[22] Y.S. Chun, E. Choi, G.T. Kim, M.J. Lee, M.J. Lee, S.E. Lee, M.S. Kim, J.W. Park, Zinc induces the accumulation of hypoxia-inducible factor (HIF)-1alpha, but inhibits the nuclear translocation of HIF-1beta, causing HIF-1 inactivation, Biochem. Biophys. Res. Commun. 268 (2000) 652–656.
[23] B. Keith, R.S. Johnson, M.C. Simon, HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression, Nat. Rev. Cancer 12 (2011) 9–22.