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SIRT1 deacetylates and stabilizes hypoxia-inducible factor-1α (HIF-1α) *via* direct interactions during hypoxia

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ABSTRACT

Upon shift to a hypoxic environment, cellular HIF-1α protein is stabilized, with a rapid decline in oxygen-sensitive hydroxylation. Several additional post-translational modifications of HIF-1α are critical in controlling protein stability during hypoxia. In the present study, we showed that SIRT1 stabilizes HIF-1α *via* direct binding and deacetylation during hypoxia. SIRT1 depletion or inactivation led to reduced hypoxic HIF-1α accumulation, accompanied by an increase in HIF-1α acetylation. Impaired HIF-1α accumulation was recovered upon inhibition of 26S proteasome activity, indicating that SIRT1 is essential for HIF-1α stabilization during hypoxia. Consistently, HIF-1α accumulation was enhanced upon over-expression of wild-type SIRT1, but not its dominant-negative form. SIRT1-mediated accumulation of HIF-1α protein led to increased expression of HIF-1α target genes, including VEGF, GLUT1 and MMP2, and ultimate promotion of cancer cell invasion. These findings collectively imply that hypoxic HIF-1α stabilization requires SIRT1 activation. Furthermore, SIRT1 protection of HIF-1α from acetylation may be a prerequisite for stabilization and consequent enhancement of cell invasion.

1. Introduction

Hypoxia-inducible factor-1α (HIF-1α) is central to the regulation of O_2 homeostasis and redox-sensitive metabolism, facilitating effective adaptation to hypoxia [1–3]. HIF-1α has a significant impact on normal physiology, and additionally influences the development of hypoxia-associated diseases, including cancer, ischemia, erythropoiesis, and osteoarthritis [2]. To facilitate rapid adjustment to a hypoxic environment, HIF-1α becomes stabilized after a fast decline in the level of oxygen-requiring hydroxylation catalyzed by HIF hydroxylase [4]. Inefficient HIF-1α hydroxylation, in turn, relaxes protein binding to the von Hippel-Lindau (VHL) protein, preventing HIF-1α degradation [5,6]. Cancer cells acquire aggressive characteristics, such as increased glucose uptake as well as invasive and angiogenic functions, through stabilizing HIF-1α protein [2,3]. Hypoxic switching in the respiratory tract is followed by alterations in the cellular redox state, and ultimately, changes in the activities of redox-sensitive metabolic pathways [1,7,8].

In recent years, regulation of HIF-1α activity by SIRT family members has been a topic of considerable interest [9–15]. Within the SIRT family, SIRT1, SIRT2, SIRT3, and SIRT6 have been identified in association with HIF-1α. SIRT3 and SIRT6 have been shown to affect HIF-1α stability *via* regulation of ROS and oxygen levels, respectively [13–15], while SIRT2 increases HIF-1α binding to prolyl hydroxylase, and subsequent hydroxylation and ubiquitination of the protein [12]. However, the mechanism by which SIRT1 regulates HIF-1α activity is a subject of controversy. The report that SIRT1 has an impact on HIF-2α [9] was a noticeable first study, leading to the discovery that the SIRT family regulates hypoxic signaling with diverse redox-sensitive mechanisms. In this early evaluation, the authors did not detect SIRT1 binding to HIF-1α protein or regulation of HIF-1α transcription, even though they examined both HIF-2α and HIF-1α. However, this preliminary

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finding was not in keeping with results from a subsequent study, which showed that SIRT1 negatively controls HIF-1α *via* formation of a physical complex between the proteins [10]. The paradoxical conclusions that SIRT1 has no impact on HIF-1α or functions as a negative regulator of HIF-1α were opposite to yet another recent report that SIRT1 positively regulates HIF-1α through stabilizing the protein [11]. These prior studies utilized systematic and reasonable approaches to reach the above conclusions within their experimental scopes. However, the question of whether SIRT1 functions as a positive or negative regulator of HIF-1α or controls HIF-1α with dual modes according to experimental conditions remains to be elucidated.

In the present study, we carefully re-evaluated the regulatory effects of SIRT1 on HIF-1α activity. Our experiments showed that SIRT1 stabilizes HIF-1α *via* direct interactions, consistent with the findings of Laemmle et al. [11]. Since SIRT1 inactivation or depletion led to impaired accumulation of HIF-1α protein without changes in the mRNA level during hypoxia, we propose that SIRT1 is an essential component for hypoxic HIF-1α stabilization. In addition, SIRT1 should protect HIF-1α against acetylation if the protein is stabilized. Experiments within the scope of the present study mainly dealt with SIRT1 regulation of HIF-1α protein stability, but not transcriptional activity. Thus, we could not define reasons underlying discrepancies between our and prior conflicting conclusion that SIRT1 negatively controls HIF-1α activity through transcriptional regulation [10].

2. Materials and methods

2.1. Cell culture and reagents

HeLa, Hep3B, HT1080, SK-Hep-1 and SiHa cells were cultured in Minimal Essential Medium (MEM; Cat. no. LM007-7, Welgene, Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (FBS; Cat. no. 43640, JRS, CA) and 1% (w/v) penicillin/streptomycin. HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Cat. no. LM001-05, Welgene) with 10% (v/v) FBS and antibiotics. H82, H221, SUN398 and SUN878 cells were maintained in RPMI 1640 (Cat. no. LM001-01, Welgene) supplemented with 10% (v/v) FBS. Cell lines were cultured in air with 5% CO_2 at 37 °C. For culture under hypoxic conditions, cells were incubated in a hypoxic chamber (Forma Anaerobic System; Thermo Scientific, MA) with 5% CO_2/1.0% O_2 and 94.0% N_2 (all v/v), respectively. The reagents used in this study were nicotinamide (NAM; Cat. no. z40206, Sigma, MO), MG132 (Cat. no. C2211, Sigma), $CoCl_2$ (Cat. no. C8661, Sigma), BML-210 (Cat. no. GR330, BIOMOL, PA), and EX-527 (Cat. no. 2780, Tocris, Bristol, UK).

2.2. Gene silencing and overexpression

For transient silencing, cells were transfected with Lipofectamine RNAi MAX (Cat. no. 13778-150, Invitrogen, CA) according to the manufacturer's instructions, using negative control or target siRNA. Oligonucleotides employed in siRNA experiments were as follows: control siRNA (Cat. no. 12935-300; Invitrogen) and SIRT1-siRNA (#1, 5′-ACUUUGCUGUAACCCUGUA-3′; #2, 5′-AGAGUUGCCACCCACACCU-3 and #3, 5′-AGAGUUGCCACCCACACCU-3′; #3, 5′-AACCUUUGCCUCAUCUGCAUU-3′). For stable silencing, the sh-SIRT1 plasmid was constructed using the forward primer (5′-caccaCACCAGATTCTTCAGTGATTGTCAtctcTGACAATCACTGAAGAATCTGGTGG-3′) and reverse primer (5′-aaaaCCACCAGATTCTTCAGTGATTGTC Agaga TGACAA TCACTGAAGAATCTGGTGG-3′) spanning the SIRT1 region. Amplified products were cloned into the pENTP™/H1/TO vector. Stable HeLa cell lines depleted of SIRT1 were established by transfection with SIRT1-shRNA or control-shRNA, followed by selection with 100 μg/mL zeocin (Invitrogen). Cells overexpressing wt-SIRT1 or the dominant-negative form of SIRT1, SIRT1/H363Y [16,17], were generated by retroviral transduction and selection with puromycin (1 μg/mL).

2.3. Western blot and acetylation assays

Cultured cells were lysed in TNN buffer containing 40 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM sodium fluoride, and protease inhibitor cocktail tablets (Cat. No. 04 693 159 001, Roche, Penzberg, Germany). Cell extracts were separated using SDS-PAGE and transferred to nitrocellulose membranes. Membranes were subsequently immunoblotted with anti-SIRT1, anti-c-Myc, anti-GST, anti-MMP2, anti-β-Actin (Cat. no. sc-55404, sc-40, sc-53909, sc-10736, sc-47778, Santa Cruz Biotechnology, CA), or anti–HIF–1α (Cat. no. 610959, BD Biosciences, NJ), followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary anti-mouse IgG (H^+L)-HRP or anti-rabbit IgG (H^+L)-HRP (Cat. no. K0211589, K0211708, Koma, Seoul, Korea) antibodies. For chemiluminescent detection, the ECL system was employed (Cat. no. sc-204806, Santa Cruz Biotechnology). For detection of acetylation of both endogenous and exogenous HIF-1α, lysates were prepared at the indicated time-points from cells exposed to hypoxia. Lysates were subjected to immunoprecipitation with anti–HIF–1α (Cat. no. NB100-105, Novus Biologicals, CO) to measure acetylation levels of endogenous HIF-1α. Acetylated HIF-1α was detected by immunoblotting with a specific anti-acetyl-lysine (AcK) antibody (Cat. no. 9441, Cell Signaling, MA). The extent of HIF-1α acetylation was quantitated by calculating the ratio of acetylated HIF-1α/HIF-1α band intensities.

2.4. Protein–protein interaction assays

Cells were lysed with NP-40 lysis buffer, followed by preclearing using normal mouse IgG (Cat. no. sc-2025, Santa Cruz Biotechnology). To detect interactions between HIF-1α and SIRT1, lysates were immunoprecipitated with either normal rabbit IgG (Cat. no. sc-2027, Santa Cruz Biotechnology), normal mouse IgG, anti-SIRT1 or anti–HIF–1α antibody (Cat. no. NB100-105, Novus Biologicals). To identify the SIRT1 binding domain of HIF-1α, HIF-1α-GST_N2, HIF-1α-GST_ODD, HIF-1α-GST_ID or HIF-1α-GST_C-TAD expression plasmids [18] were cotransfected with Myc-tagged SIRT1. Immunoprecipitation was performed as described above, using an anti-GST antibody.

2.5. RT-PCR

Total RNA was extracted using an RNeasy Mini kit (Cat. No. 74106, Qiagen, GmBH, Germany). For cDNA synthesis, total RNA was reverse-transcribed with an iScript cDNA synthesis kit (Cat. No. 170-8890, Bio-Rad). Reverse transcriptase-polymerase chain reaction was performed using a Maxime PCR PreMix kit (i-StarTaq; Cat. No. 25167, iNtRON Biotechnology, SungNam, Korea). The following primer sequences were used for semi-quantitative RT-PCR: HIF-1α forward (5′-CTCAAAGTCGGACAGCCTCA-3′) and reverse (5′-CCCTG CAGTAGGTTTCTGCT-3′); SIRT1 forward (5′-CAAACTTTGCTGTAACCC TGT-3′) and reverse (5′-CAGCCACTGAAGTTCTTTCAT-3′); VEGF forward (5′-TGCATT CACATTTGTTGTGC-3′) and reverse (5′-AGACCCTGGTGGACATCTTC-3′); Glut1 forward (5′-TGTCCTATCT-GAGCATCGTG-3′) and reverse (5′-CTCCTCGGGTGTCTT ATCAC-3′); β-actin forward (5′-AAGGATTCCTATGTCGGC-3′) and reverse (5′-CTTCATGATGGAGTTGAAGGT-3′).

2.6. Real-time PT-PCR

Real-time PCR was performed using the KAPA SYBR FAST Universal qPCR kit (Cat. No. KK4602, Kapa Biosystems, MA) and the CFX96 real-time PCR detection system (Cat. No. 185-5096, BioRad, CA). The primer sequences used were: HIF-1α forward (5′-CTCAAA GTCGGACAGCCTCA-3′) and reverse (5′-CCCTGCAGTAGGTTTCTGCT-3′); SIRT1 forward (5′-CAAACTTTGCTGTAACCCTGT-3′) and reverse (5′-CAGCCACTGA AGTTCTTTCAT-3′); VEGF forward (5′-TGCATTCA-CATTTGTTGT GC-3′) and reverse (5′-AGACCCTGGTGGACATCTTC-3′); Glut1 forward (5′-TGTCCTATCTGAGCATCGTG-3′) and reverse (5′-CTCCTCGGGTGTCTTATCAC-3′); β-actin forward (5′-AAGGATT CCT ATGTCGGC-3′) and reverse (5′-CTTCATGATGGAGTTGAAGGT-3′). Each RT-PCR reaction was repeated at least three times to demonstrate reproducibility, and data analyzed using the CFX96 Real-Time System. Normalized values were obtained by subtracting the threshold cycle (Ct) of *β-actin* from the Ct values of target genes, yielding ΔCt values, and the ΔΔCt formula used as an indication of the relative transcriptional level.

2.7. Invasion assay

Cell invasion activity was analyzed using a transwell chamber (Cat. no. 3422, Corning, NY) in which inserts were coated with dilutions of BD Matrigel Matrix Growth Factor Reduced (Cat. No. 356234, BD Biosciences, Bedford, MA) [19]. SIRT1-depleted HeLa cells transfected with plasmids encoding wt–HIF–1α were seeded into the upper wells at a density of 1×10^4 cells per well in serum-free MEM, and incubated for 24 h under either normoxic or hypoxic conditions. Cells invading the lower surface membrane were fixed and stained with Hemacolor solution (Cat. No. 1.11661, Merck, Darmstadt, Germany). The extent of invasion was determined by counting the number of mobile cells under a light microscope.

3. Results

3.1. SIRT1 activation is required for hypoxic accumulation of HIF-1α protein

Three different results have been reported in association with SIRT1 regulation of HIF-1α: SIRT1 has no effect [9] or serves as a negative [10] or positive regulator [11]. To delineate the factors underlying these discrepancies, we carefully examined the influence of SIRT1 on HIF-1α in a variety of cell lines under hypoxia, including those used in prior reports. Our data indicate that SIRT1 positively controls HIF-1α activity, consistent with the findings of Laemmle et al. [11]. SIRT1 depletion using SIRT1-siRNA or -shRNA led to significant inhibition of HIF-1α accumulation in HeLa cells during hypoxia (Fig. 1A and B). Three different SIRT1-siRNAs

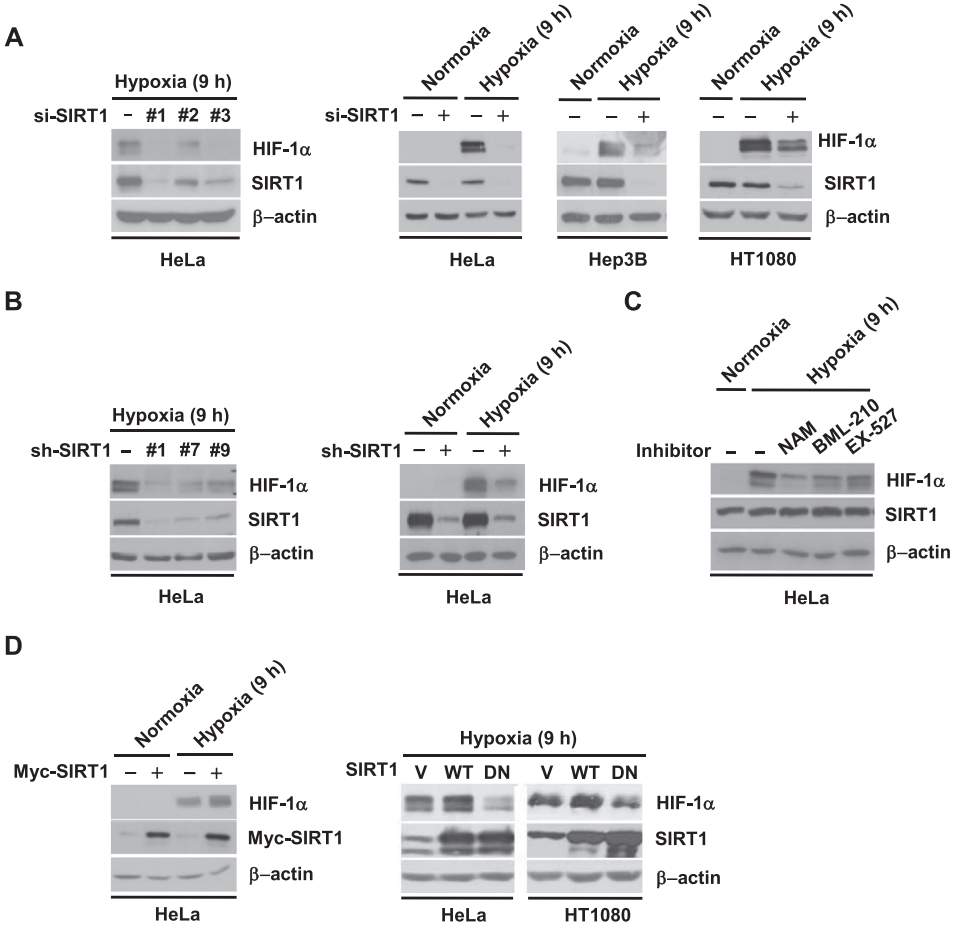

Fig. 1. SIRT1 promotes hypoxic HIF-1α accumulation. (**A**) HIF-1α protein accumulation was monitored in HeLa, Hep3B and HT1080 cells under normoxic and hypoxic conditions after SIRT1 depletion using siRNAs targeting SIRT1 mRNA (left panel, three types of siRNA #1, #2, and #3; right panel, siRNA #1). – indicates control siRNA. (**B**) HIF-1α protein accumulation was monitored in established HeLa clones (#1, #7, and #9) defective in SIRT1 by virtue of stable incorporation of shRNAs under hypoxic conditions for 9 h or normoxia. – indicates control shRNA. (**C**) HIF-1α protein levels were compared among cell lines exposed to hypoxia for 9 h and treated with SIRT1 inhibitors, NAM (20 mM), BML-210 (10 μM) or EX-527 (1 μM). (**D**) HIF-1α protein levels were evaluated in HeLa cells overexpressing wt-SIRT1 under normoxic and hypoxic conditions (left panel). After 9 h of incubation under hypoxia, HIF-1α protein levels were evaluated in cells overexpressing wild-type SIRT1 (WT) or dominant-negative SIRT1/H363Y(DN) (right panel).

exerted similar inhibitory effects on HIF-1α accumulation (left panel, Fig. 1A). Impaired HIF-1α accumulation during hypoxia was similarly noted in two other cell lines, Hep3B [9,20] and HT1080 [10], employed in prior reports that presented inconsistent data (right panel, Fig. 1A). Reduced accumulation was additionally found in SIRT1-depleted clonal HeLa cells stably incorporating SIRT1-shRNA (Fig. 1B). However, in contrast to hypoxic conditions, SIRT1 depletion did not affect HIF-1α accumulation under normoxia (Fig. 1A and B), indicating no impact of SIRT1 on normoxic degradation of HIF-1α. Our findings clearly imply that SIRT1 is required for HIF-1α accumulation during hypoxia, but not normoxia. Next, we examined the effects of SIRT1 inhibitors on HIF-1α accumulation. Notably, SIRT1 inhibitors, such as nicotine adenine amide (NAM), BML-210, and EX-527 (Fig. 1C and Supplementary Fig. 1), as well as dominant-negative SIRT1, SIRT1/H363Y (right panel, Fig. 1D and Supplementary Fig. 1), suppressed HIF-1α accumulation during hypoxia. Consistent with these findings, the hypoxic HIF-1α level was increased under conditions of wt-SIRT1 overexpression (Fig. 1D). The collective results obtained using dominant-negative SIRT1 and specific inhibitors indicate that SIRT1 activity, rather than protein expression *per se*, is essential for HIF-1α accumulation during hypoxia.

3.2. SIRT1 activation is required for hypoxic stabilization of HIF-1α

Hypoxic HIF-1α accumulation is controlled *via* transcription and post-translational stabilization [21]. Normoxic HIF-1α degradation is attenuated after commencement of hypoxia [21]. To determine whether SIRT1-induced promotion of HIF-1α accumulation is attributable to stabilization, we measured the effects of SIRT1 depletion under conditions where HIF-1α degradation is inhibited. Treatment with MG132, a potent inhibitor of 26S proteosomal proteolysis [22], rescued accumulation of HIF-1α protein that was reduced following SIRT1-siRNA treatment in HeLa and HT1080 (left panel, Fig. 2A) or SIRT1 inactivation in HeLa cells (right panel, Fig. 2A). In contrast, the HIF-1α mRNA level was not influenced by SIRT1 depletion (Fig. 2B). In view of these results, we propose that SIRT1 activation induces stabilization of HIF-1α by preventing protein degradation during hypoxia. Our findings are in keeping with those of Laemmle et al. [11], but not the initial two reports [9,10]. We further examined the reproducibility of the current findings using other cell lines. Similar to HeLa, HT1080 and Hep3B cells, all cancer cell lines examined, including SK-Hep-1, SiHa, H82, H441, SNU398 and SNU878, exhibited impaired accumulation of HIF-1α protein when SIRT1 was inactivated using the inhibitor, NAM (Fig. 2C). Our data collectively imply that this finding is not

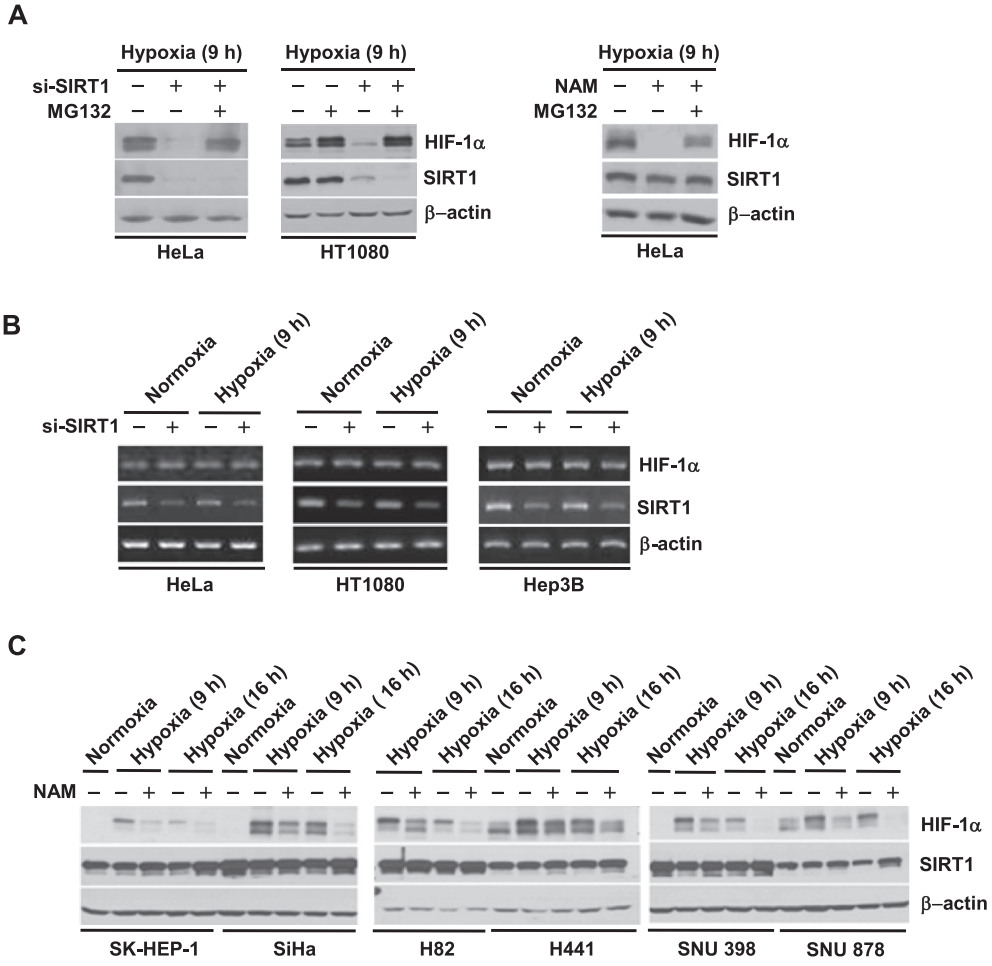

Fig. 2. SIRT1 controls hypoxic HIF-1α stabilization. (**A**) HIF-1α degradation was inhibited by MG132 (20 μM) in HeLa and HT1080 cells transfected with either SIRT1-siRNA (+) or control siRNA (−) (left panel), and HeLa cells treated with NAM (right panel) under hypoxic exposure for 9 h. **(B)** At 9 h after commencement of hypoxia, HIF-1α mRNA levels were evaluated *via* quantitative RT-PCR with or without SIRT1 depletion achieved using SIRT1 (+) and control siRNA (−), respectively. **(C)** HIF-1α protein levels under hypoxic conditions were compared in human cancer cell lines with and without SIRT1 inhibition using 20 mM NAM.

cell line-specific, but general. Thus, we propose that SIRT1 activation is essential for stabilization of HIF-1α by preventing its degradation during hypoxia.

3.3. SIRT1 interacts directly with HIF-1α

A preliminary study showed no binding of SIRT1 with HIF-1α [9], but interactions were subsequently detected [10,11]. To resolve this discrepancy, we carefully examined the interactions between the two proteins using previously employed cell lines. Initially, we evaluated interactions in 293T cells transiently cotransfected with both SIRT1 and HIF-1α. Immunoprecipitation of SIRT1 protein resulted in co-precipitation of HIF-1α (Fig. 3A). Reciprocal tests demonstrated co-precipitation of SIRT1 in a complex immunoprecipitated with anti–HIF–1α. Interactions were confirmed using immunoprecipitates of HIF-1α that endogenously accumulated upon exposure of HeLa and HT1080 cells to hypoxic conditions. This was reciprocally confirmed with experiments employing immunoprecipitates of SIRT1. Further analysis of these interactions verified that SIRT1 physically binds to the inhibitory domain (ID) of HIF-1α (Fig. 3B and Supplementary Fig. 2) that is associated with suppression of transcriptional activation [23]. The present data support the reports of Lim [10] and Laemmle [11].

3.4. SIRT1 protects against HIF-1α acetylation during hypoxic stabilization

Two distinct effects of SIRT1 on HIF-1α activity have been reported: physical interactions inducing either transcriptional suppression [10] or activation due to stabilization of the protein [11]. In the present study, we hypothesized that if SIRT1 directly controls HIF-1α acetylation, its inactivation should induce acetylation, leading to HIF-1α instability. As predicted, SIRT1 depletion (Fig. 3C) or inhibition of SIRT1 action (Fig. 3D) enhanced HIF-1α acetylation, accompanied by degradation under hypoxic conditions. SIRT1 inactivation-induced acetylation and subsequent reduction of HIF-

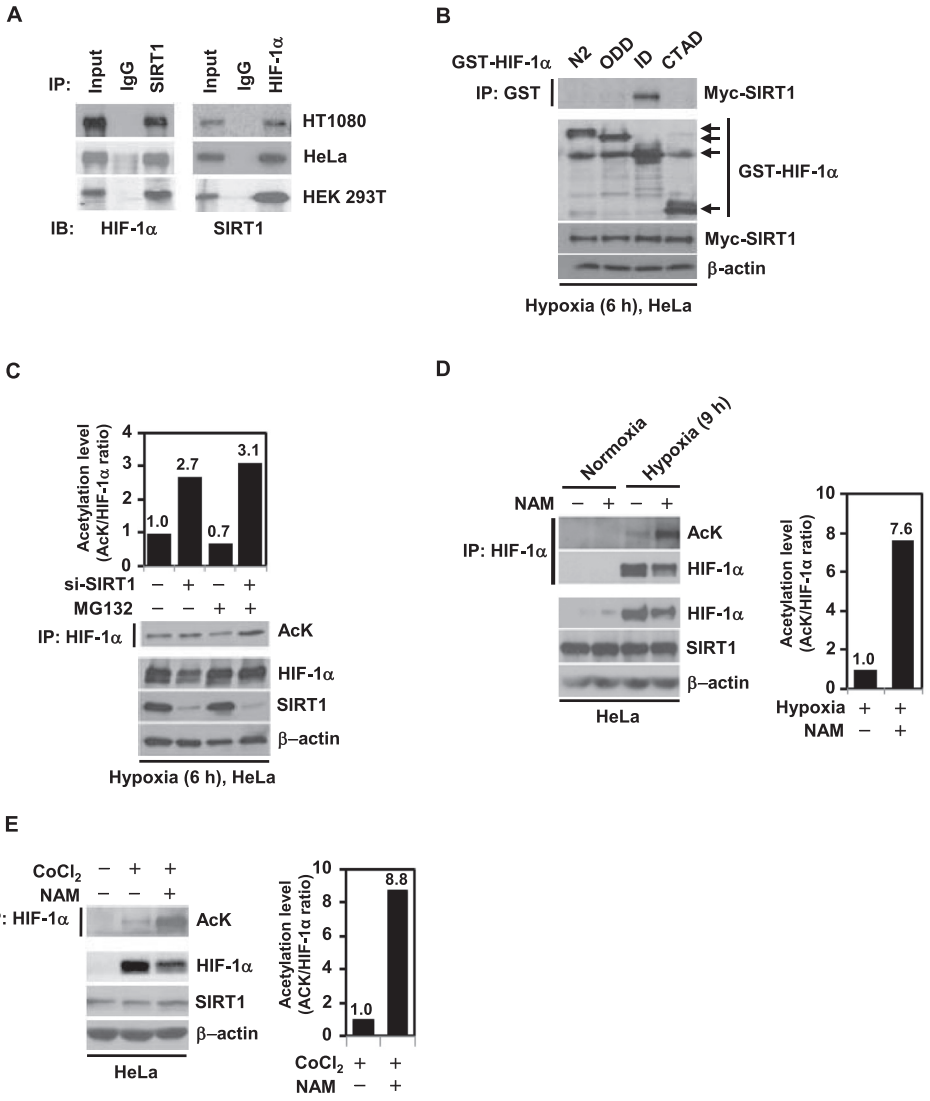

Fig. 3. SIRT1 binds and deacetylates HIF-1α. (**A**) 293T cells cotransfected with SIRT1 and HIF-1α were employed for immunoprecipitation experiments, along with HeLa and HT1080 cells exposed to 6 h of hypoxia. Immunoprecipitated SIRT1 and HIF-1α from cells were reciprocally probed with anti–HIF–1α (left) and anti-SIRT1 antibodies (right), respectively. (**B**) HeLa cells were transiently cotransfected with GST-fused forms of each indicated domain, together with a myc-tagged form of SIRT1. Immunoprecipitated domains with antibodies against GST were probed with an anti-myc antibody. Arrows indicate the positions of GST-fused HIF-1α proteins. (**C** and **D**) Acetylation of immunoprecipitated HIF-1α was assessed in HeLa cells in the presence of SIRT1-siRNA (**C**) or NAM (**D**). (**E**) Acetylation of immunoprecipitated HIF-1α was assessed in HeLa cells in which SIRT1 inactivation was achieved by the addition of NAM under hypoxia-mimicking conditions accomplished by $CoCl_2$ supplementation for 9 h.

1α was additionally observed in cells under hypoxia-mimicking conditions induced by treatment with $CoCl_2$ (Fig. 3E). In contrast, SIRT1 depletion under normoxic conditions affected neither acetylation nor the level of HIF-1α (Supplementary Fig. 3). Our results showing increased HIF-1α acetylation during protein destabilization *via* either SIRT1 inhibition or depletion support the theory that SIRT1-mediated deacetylated HIF-1α is stable under hypoxic conditions. Hence, for stabilization of HIF-1α during hypoxia, it appears that SIRT1 protects against acetylation.

3.5. SIRT1 positively controls hypoxic invasion of tumor cells via HIF-1α

To determine whether SIRT1-mediated stabilization of HIF-1α has biological consequences, we initially examined the expression levels of downstream target molecules controlled by HIF-1α. Among the HIF-1α targets, Glut-1, VEGF and MMP2 involved in glucose transport, angiogenesis, and invasion, respectively, were selected [1–3]. Initial semi-quantitative RT-PCR analysis in HeLa cells disclosed increased levels of Glut-1 and VEGF mRNA during hypoxia, which were inhibited upon SIRT1 depletion (Fig. 4A). Quantitative assessment using real-time RT-PCR demonstrated that the 1.97- and 3.21-fold increase in Glut-1 and VEGF expression was reduced to 1.61 and 1.77-fold upon SIRT1 depletion, respectively (Fig. 4B). Increased MMP2 secretion detected during hypoxia was also reduced following SIRT1 depletion (Fig. 4C). As expected based on the reduction in MMP2 expression, SIRT1 depletion suppressed the rate of invasion that had increased upon exposure of cells to hypoxia (Fig. 4D). Introduction of HIF-1α resulted in significant elevation of invading cells under conditions without SIRT1 depletion, while under SIRT1-depleted conditions, this increase was slight. Accordingly, we propose that SIRT1 promotes invasion of cancer cells *via* stabilization of HIF-1α during hypoxia.

4. Discussion

HIF-1α stabilization is essential for cellular adaptation to environmental shifts to oxygen-deficient conditions. Our present findings demonstrate that under hypoxic conditions, SIRT1 stabilizes HIF-1α *via* direct binding and deacetylation, but has no impact on normoxic degradation of HIF-1α protein.

The regulatory effects of SIRT1 on HIF transcriptional activity have been explored in three previous studies [9–11]. Among these, data from Laemmle et al. [11] are in line with our present findings. In contrast to our observation that SIRT1 positively regulates HIF-1α activity *via* deacetylation and consequent stabilization, SIRT1 was initially reported to have either no impact on [9] or negatively regulate HIF-1α activity [10]. Notably, the two earlier reports did not explore the effects of SIRT1 on HIF-1α stability, although HIF-1α stabilization is a critical step that occurs prior to HIF-1α-mediated transcription of relevant genes. To further investigate this issue, we employed several cell lines, including Hep3B and HT1080 used earlier. In our experiments, these cell lines failed to show HIF-1α accumulation upon SIRT1 depletion or inactivation, while SIRT1 overexpression resulted in increased HIF-1α protein levels. In contrast, the HIF-1α mRNA level was not affected by SIRT1. Additionally, inhibition of proteasome activity with MG132 led to the recovery of HIF-1α protein levels that had been suppressed upon

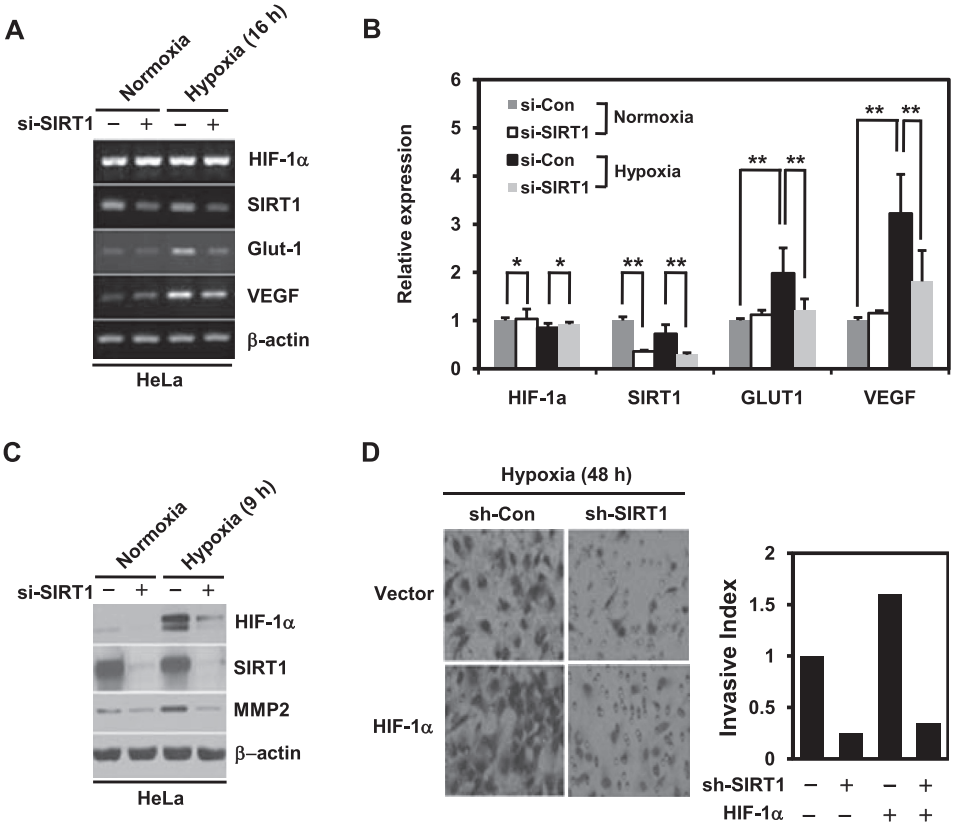

Fig. 4. SIRT1 promotes target gene expression and invasion of cancer cells. (**A** and **B**) At 16 h after commencement of hypoxia in HeLa cells, the levels of HIF-1α and its transcriptional targets were evaluated using quantitative RT-PCR (**A**), real-time PCR (**B**) and Western blot (**C**), with or without SIRT1 depletion achieved with SIRT1-or control siRNA, respectively. (**D**) Invasion rates of HeLa cell clones with stable incorporation of sh-SIRT1 (+) or control shRNA (–) were evaluated with and without HIF-1α transduction after exposure to hypoxic conditions for 48 h.

SIRT1 depletion. These findings imply that SIRT1 is required for stabilization of HIF-1α. Data from the two earlier reports must be interpreted with caution, as neither study considered HIF-1α stability. In fact, investigators focusing on SIRT1 regulation of HIF-2α detected neither SIRT1 binding to HIF-1α nor regulation of HIF-1α [9]. However, these results were challenged by another research group reporting that SIRT1 interacts with and controls HIF-1α transcription *via* deacetylation at Lys674 [10]. In the present study, we observed that SIRT1 interacts with and deacetylates HIF-1α. Furthermore, SIRT1-induced deacetylation of HIF-1α prevented protein degradation, leading to eventual increase in target gene expression (Glut1, VEGF, and MMP2) and consequent cancer cell invasion during hypoxia, in contrast to the earlier finding that SIRT1 deacetylation of HIF-1α suppresses its transcriptional activity [10].

The earlier conclusion that SIRT1 suppresses HIF-1α transcriptional activity appears reasonable, based on previous observations that SIRT1 generally affects the activities of transcription factors, mainly leading to suppression of transcription [24]. This reference was further supported by subsequent studies demonstrating SIRT1-mediated regulation of HIF-1α activity including HIF-2α [9,10]. Therefore, it is possible that SIRT1 exerts a dual regulatory function, i.e., promotes HIF-1α stabilization and suppresses transcriptional activity. Further research is warranted to resolve the complex biological interactions between SIRT1 and HIF-1α.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.119.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.119.

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