Overexpression of SIRT1 prevents hypoxia-induced apoptosis in osteoblast cells

LU ZHOU[1*], SUNG IL WANG[2*], YOUNG JAE MOON[3], KYOUNG MIN KIM[4], KWANG BOK LEE[2], BYUNG-HYUN PARK[3], KYU YUN JANG[4] and JUNG RYUL KIM[2]

[1]Department of Sports Medicine, Taishan Medical University, Taian, Shandong 271000, P.R. China; Departments of [2]Orthopaedic Surgery, [3]Biochemistry and [4]Pathology, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, Jeonju 54896, Republic of Korea

Received August 31, 2016; Accepted April 28, 2017

DOI: 10.3892/mmr.2017.6917

Abstract. Hypoxic-ischemic injury of the bone results in osteonecrosis. Nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase sirtuin-1 (SIRT1), a type of NAD-dependent deacetylase, is involved in multiple biological functions, particularly in anti-apoptosis. However, the effects of SIRT1 in osteoblasts remain unclear and whether SIRT1 protects osteoblasts in hypoxic conditions remains to be elucidated. In the present study, the role of SIRT1 in the osteoblast cells under hypoxia and the underlying mechanism of the anti-apoptotic activity of SIRT1 were investigated. MC3T3-E1 osteoblast cells were used for the present study and oxygen-absorbing packs were used to induce cell hypoxia and apoptosis. MC3T3-E1 cells were overexpressed SIRT1 by transfection with a SIRT1 adenovirus. The small interfering RNA of SIRT1 to was used to transfect cells to decrease the protein level. An MTT assay was used to estimate cell viability. Apoptosis was examined with the APOPercentage apoptosis assay kit and the activity of caspases was measured by a caspase 3 and 7 activity kit. Co-immunoprecipitation was used to investigate protein binding ability. The mRNA and protein expression levels were quantified with reverse transcription-quantitative polymerase chain reaction and immunoblotting. It was demonstrated that the expression of SIRT1 mRNA and protein were elevated, and peaked at 12 h under hypoxic conditions. The data demonstrated that SIRT1 overexpression in cells significantly increased cell viability and markedly decreased the percentage of apoptosis compared with the control and knockdown groups. Furthermore, overexpression of SIRT1 significantly activated anti-apoptotic effects by deacetylating lysine residue binding to protein kinase B and decreasing the activity of caspases 3, 9 and subsequent pathways. The results from the present study suggested that SIRT1 may serve a protective function in hypoxia-induced apoptosis in MC3T3-E1 cells, and that SIRT1 intervention may potentially aid in the treatment of ischemic bone disease.

Correspondence to: Professor Jung Ryul Kim, Department of Orthopaedic Surgery, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, 567 Baekje-daero, Deokjin-gu, Jeonju 54896, Republic of Korea
E-mail: jrkeem@jbnu.ac.kr

*Contributed equally

Key words: nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1, hypoxia, protein kinase B, apoptosis, osteoblast

Introduction

Ischemic osteonecrosis of the femoral head commonly leads to the destruction of the hip joint and is a progressive, debilitating disease (1). Multiple factors may result in the occurrence of the disease, including fracture and dislocation, glucocorticoid and radiation therapy, alcoholism, connective tissue disease, or infection (2). The key elements in the pathogenesis of ischemic necrosis of the femoral head are vascular disruption and defective bone remodeling. Vascular disruption normally induces cell ischemia by hypoxia. The hypoxic condition normally induces apoptosis in osteoblasts, vascular endothelial cells and smooth muscle cells by triggering mitochondrial permeability. A previous study demonstrated that hypoxia activated a mitochondrial-dependent classic apoptosis pathway, which increased cytochrome *c* release and the subsequent cleaving and activation of caspase 9 and other effector caspases (3).

Sirtuins (SIRTs), which were first identified in yeast, are nicotinamide adenosine dinucleotide (NAD)-dependent deacetylases. Currently, seven SIRTs have been identified and are expressed in mammalian cells. SIRT1, SIRT6 and SIRT7 are localized in the nucleus, SIRT2 in the cytoplasm and SIRT3, SIRT4 and SIRT5 are expressed in the mitochondria (4,5). SIRT1 affects a series of biological functions, including DNA repair, tumor suppression, energy metabolism and mitochondrial permeability. SIRT1 controls longevity and delays senility in lower organisms and mammals (6). Previous studies have demonstrated that SIRT1 has vital inhibitory effects in chondrocytes and on cardiac myocyte apoptosis in vascular

endothelial cells and mesenchymal stem cells (7-9). These effects are primarily mediated by deacetylating substrates, including important proteins, transcription factors or co-factors, including p53, protein kinase B (Akt), the Forkhead box O (FoxO) family and β-catenin, a co-activator of canonical Wnt signaling. The deacetylation of these proteins by SIRT1 inhibits the apoptosis process in mammalian cells (10-12). Therefore, it is possible that SIRT1 serves an important role in cell defence and survival through interaction with apoptosis-inducible nuclear proteins. Due to the protective effects of SIRT1 in cells, SIRT1 is additionally a key regulator of aging and apoptosis in osteoblasts. Osteoblasts, which are the primary functional cells for bone formation, are responsible for the synthesis, secretion and mineralization of bone matrix. Any factor that suppresses the generation and differentiation or promotes the apoptosis of osteoblasts may reduce bone formation and result in an imbalance in bone remodeling, leading to bone loss. Thus hypoxia-induced apoptosis and necrosis interfere with the bone remodelling process. To the best of our knowledge, no studies have reported the direct role of SIRT1 in hypoxia-induced apoptosis of osteoblasts.

The present study investigated the protective effect of SIRT1 in the survival of MC3T3-E1 osteoblast cells against a hypoxic stimulus, and attempted to identify the associated underlying mechanism. The results of the present study demonstrated that SIRT1 deacetylases the substrates of phosphoinositide 3-kinase (PI3K)/Akt and nuclear factor (NF)-κB in downstream anti-apoptosis pathways and serves an important role in mediating the anti-apoptotic effects in MC3T3-E1 cells.

Materials and methods

Cell culture and transfection. MC3T3-E1 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and were plated in 6-well plates at a density of $1.5x10^6$ cells/well in Minimum Essential α-Medium (αMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 10 units/ml penicillin and 10 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). For the SIRT1 silencing experiment, transfection of MC3T3-E1 cells was performed by using small interference (si)RNA. The siRNA sequences that were used were as follows: si-SIRT1 sense, 5'-GAGACUGCGAUG UCAUAAUTT-3' and antisense, 5'-AUUAUGAC AUCGCA GUCUCTT-3'; and scrambled siRNA sense, 5'-CGCUCC GAA CGUGCUACGUTT-3' and antisense, 5'-ACGGUACAC GUUCAAAGAATT-3'. siRNAs were purchased from GE Dharmacon (Lafayette, CO, USA). Transient transfection was performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 6 h using a final concentration of siRNAs of 5 nM, according to the manufacturer's protocol). For the SIRT1 overexpression experiment, MC3T3-E1 cells were transfected for 8 h at 37˚C with $1x10^9$ particle forming units of SIRT1 adenovirus, which was obtained from Professor B.H. Park (Department of Biochemistry, Chonbuk National University, Jeonju, Republic of Korea). Viruses were purified from the supernatants of 293 cell cultures by cesium chloride density gradient centrifugation at 70,000 x g at 4˚C for 3 h. As a control, the pAd/CMV/V5-GW/lacZ vector (Invitrogen; Thermo Fisher Scientific, Inc.) was used to produce LacZ-bearing adenovirus. MC3T3-E1 cells were incubated in α-MEM medium without antibiotics for 24 h prior to transfection to enhance the transfection efficiency.

Establishment of hypoxic culture conditions. MC3T3-E1 cells were incubated at 37˚C in anaerobic jars (Oxoid; Thermo Fisher Scientific, Inc.) with oxygen-absorbing packs (AnaeroGen; Oxoid; Thermo Fisher Scientific, Inc.). Prior to transferring to the hypoxic chamber, the cells were moved to a glucose-free medium. Within 0.5 h, O_2 had decreased to less than 0.1%.

Analysis of cell viability. An MTT assay was used to estimate cell viability (12,13). Briefly, cells were plated at a density of $1x10^4$ cells/well in α-MEM medium supplemented with 10% FBS and antibiotics in 96-well plates. Following exposure to hypoxia for 24 h, MTT solution (Sigma-Aldrich; Merck KGaA) was added in plates at a final concentration of 0.5 mg/ml for 3 h at 37˚C. Following removal of the medium, 100 μl dimethyl sulfoxide solution was added to dissolve the formazan crystals. The absorbance at 570 nm was detected with a microplate reader (Synergy 4 Hybrid Multi-Mode; BioTek Instruments, Inc., Winooski, VT, USA).

Detection of caspase 3 and 7 activity. Caspase 3 and 7 activity was detected using a luminescence-based Caspase-Glo® 3/7 Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Cells were cultured in hypoxia for 24 h and Caspase-Glo 3/7 reagent was added to each well in a 1:1 ratio according to the manufacturer's protocol. Following agitation for 10 min on a plate shaker at room temperature, 90% of the lysate volume was transferred to a 96-well solid-white plate. Cell lysates were analyzed with a microplate reader (Synergy 4 Hybrid Multi-Mode; BioTek Instruments, Inc.).

Detection of apoptosis. Hypoxia-induced cell apoptosis was determined using the Cell-APOPercentage™ Apoptosis Assay (cat. no. GB2356929; Biocolor Ltd., Carrickfergus, Northern Ireland). Digital images of APOPercentage dye-labeled cells, appearing bright pink against a white background under a light microscope, were used to detect apoptotic cells. To quantify the level of apoptosis, cells were incubated with a 1:100 dilution of the dye for 5 min at 37˚C following culture under each experimental condition. Cells were then washed with PBS and the dye within the labeled cells was released into the supplied dye-release reagent. The contents of each well (250 μl) were transferred to a 96-well flat-bottomed plate, and absorbance was read at 550 nm with a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The relative levels of SIRT1, tumor necrosis factor receptor-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis 2 (cIAP2) mRNA were measured using RT-qPCR, as previously described (13). Total RNA was extracted from cells at a density of $1.5x10^6$ cells/well, using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was precipitated with RNase-free chloroform and isopropanol (Sigma-Aldrich; Merck KGaA) and dissolved in diethyl pyrocarbonate-treated distilled water (Promega Corporation).

Total RNA (1 μg) free of genomic DNA contamination was reverse-transcribed into cDNA using the random hexamer primer provided in the RevertAid First Strand cDNA Synthesis kit (cat. no. K1612; Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol in a 20 μl reaction volume. The temperature protocol was as follows: At 25˚C for 5 min, at 37˚C for 60 min and at 70˚C for 5 min. Specific primers for each gene were designed using the Primer Express software version 3.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sequences used in the present study were as follows: SIRT1 forward, 5'-AAGTTG ACTGTGAAGCTGTACG-3' and reverse, 5'-TGCTACTGG TCTTACTTTGAGGG-3'; TRAF2 forward, 5'-TCCCTG GAGTTGCTACAGC-3' and reverse, 5'-AGGCGGAGCACA GGTACTT-3'; cIAP2 forward, 5'-TTTCCGTGGCTCTTA TTCAAACT-3' and reverse, 5'-GCACAGTGGTAGGAA CTTCTCAT-3'; and GAPDH forward, 5'-TGTGGGCATCAA TGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCG GGTCAAT-3'. qPCR was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR reactions were carried out in a final volume of 25 μl, containing 1 μl forward and 1 μl reverse primers (10 μM), 0.5 μl cDNA (500 nM), 0.5 μl dNTP Mix (200 μM), and 1 U (0.2 μl) GoTaq® DNA polymerase in 1X Green GoTaq® Reaction Buffer (Promega Corporation). Thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation at 94˚C for 30 sec, annealing for 45 sec at an optimised temperature for each primer pair, and extension at 72˚C for 90 sec, according to the manufacturer's protocol. Relative gene expression was quantified according to the comparative Cq method (14) and normalized to GAPDH expression. All reactions were performed in triplicate.

Western blot analysis. Cells ($1.5x10^6$) were homogenized and lysed in lysis buffer (20 mmol/l HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin; Sigma-Aldrich; Merck KGaA) on ice for 30 min. Protein concentrations were quantified using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts (20 μg) of extracted protein samples were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Following blocking in 5% skimmed milk at 4˚C for 1 h, the membranes were probed with the following primary antibodies at 4˚C for 12 h: Anti-SIRT1 (1:1,000; cat. no. 8469; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated (p)-p65 (1:1,000; cat. no. 3033; Cell Signaling Technology, Inc.), anti-acetylated-lysine (1:1,000; cat. no. 9441; Cell Signaling Technology, Inc.), anti-β-actin (1:2,000; cat. no. sc-81178; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-B-cell lymphoma 2 (Bcl-2; 1:1,000; cat. no. sc-492; Santa Cruz Biotechnology, Inc.), anti-Bcl-2 associated X protein (Bax; 1:1,000; cat. no. sc-20067; Santa Cruz Biotechnology, Inc.), anti-caspase 3 (1:1,000; cat. no. sc-1225; Santa Cruz Biotechnology, Inc.), anti-caspase 9 (1:1,000; cat. no. sc-133109; Santa Cruz Biotechnology, Inc.), anti-cleaved-caspase 3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), anti-cleaved caspase 9 (1:1,000; cat. no. 9509; Cell Signaling Technology, Inc.), anti-p-Akt (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), and anti-hypoxia inducible factor-1α (HIF-1α; 1:2,000; cat. no. ab113642; Abcam, Cambridge, MA, USA). Membranes were then incubated with the following horseradish peroxidase-conjugated secondary antibodies; goat anti-mouse immunoglobulin (Ig) G (1:5,000; cat. no. 31430; Thermo Fisher Scientific, Inc.) and goat anti-rabbit IgG (1:5,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) at 4˚C for 1 h. Protein bands were visualized using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) on an Amersham Imager 600UV system (GE Healthcare Life Sciences, Little Chalfont, UK).

Immunoprecipitation. Whole cell lysates were pre-cleared with Protein A/G plus (Santa Cruz Biotechnology, Inc.) for 30 min at 4˚C. Beads were pelleted at 1,000 x g for 30 sec at room temperature, and pre-cleared supernatants were incubated with 10-20 μg primary antibodies against SIRT1 (1:50; cat. no. 8469; Cell Signaling Technology, Inc.) and Akt (1:50; cat. no. sc-5298; Santa Cruz Biotechnology, Inc.), using normal magnetic bead-conjugated IgG (1:50; cat. no. 5873; Cell Signaling Technology, Inc.) as a negative control. The reactions were kept on a rotating wheel at 4˚C overnight. When agarose or a gel conjugate was unavailable, lysates were incubated with primary antibody or an equivalent amount of control IgG for 2 h at 4˚C and then overnight along with Protein A/G plus beads to collect the immune complexes. Beads were collected by centrifugation at 16,000 x g at 4˚C for 10 min, washed several times with radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc.), washed once with PBS, and re-suspended in SDS-PAGE sample loading buffer (Geno Technology, Inc., St. Louis, MO, USA). Immune complexes and 75-100 μg input proteins were resolved by 12% SDS-PAGE.

Statistical analysis. Data are expressed as the mean ± standard error of the mean of at least 3 independent experiments. The statistical significance of the differences between groups was assessed using one-way analysis of variance followed by a post hoc Fisher's protected least significant difference test for multiple comparisons. Statistical analysis was performed using GraphPad Prism software version 5.02 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Abnormal SIRT1 expression in hypoxia-induced apoptosis in MC3T3-E1 cells. RT-qPCR and western blot analysis were used to detect the expression level of SIRT1 in hypoxia-induced apoptosis in MC3T3-E1 cells. The differences in mRNA expression were compared with RT-qPCR at different times under hypoxia. As demonstrated in Fig. 1A, SIRT1 mRNA appeared to be elevated in the first 12 h of the experiment but was markedly downregulated in the later phase. In Fig. 1B, western blot analysis revealed a pattern of SIRT1 protein expression levels similar to the PCR data. In addition, HIF-1a protein expression appeared to be upregulated in the first 12 h of the experiment, whereas it decreased in the later phase.

SIRT1 interference affects hypoxia-induced apoptosis in MC3T3-E1 cells. To investigate the role of SIRT1 in

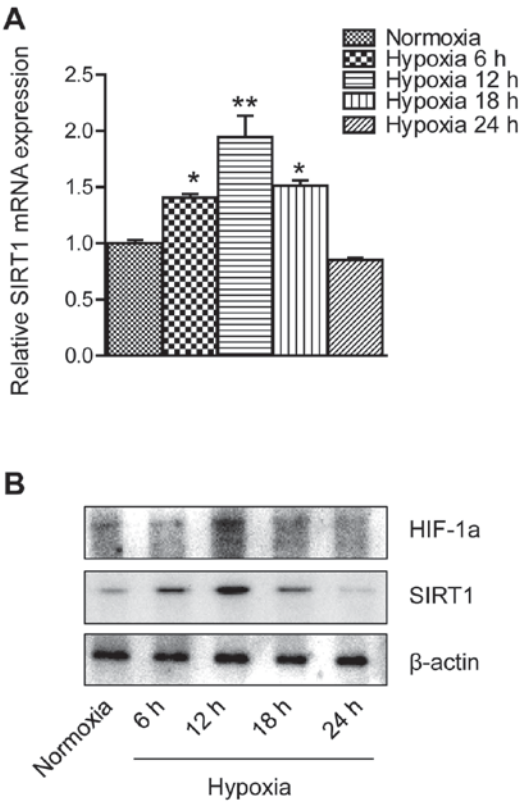

Figure 1. Expression of SIRT1 in hypoxia-induced MC3T3-E1 cell apoptosis. (A) MC3T3-E1 cells were subjected to normoxia or hypoxia for 6, 12, 18 or 24 h. SIRT1 mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. the normoxia group. **P<0.01 vs. the normoxia group. The data are presented as the mean ± standard deviation of three independent experiments. (B) MC3T3-E1 cells were treated as in (A) and SIRT1 protein expression in whole cell lysates of MC3T3-E1 cells was detected by western blotting. Each sample was analyzed in triplicate. SIRT1, nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1; HIF, hypoxia inducible factor.

hypoxia-induced apoptosis in MC3T3-E1 cells, the protein levels of SIRT1 were altered. SIRT1 was overexpressed by using an adenovirus and knocked-down by si-RNA. The western blotting data demonstrated that the protein level of SIRT1 was successfully altered by knock-down or overexpression (Fig. 2A). The data demonstrated that SIRT1 overexpression in cells significantly increased cell viability and markedly suppressed the pace of apoptosis. The activity of the caspase 3/7 pathway was nullified by SIRT1 overexpression which interrupted the process of apoptosis response to hypoxia. By contrast, SIRT1 knockdown cells demonstrated the opposite phenotype (Fig. 2B-D). Taken together, these results suggested that SIRT1 served a protective effect in hypoxia-induced apoptosis in MC3T3-E1 cells.

SIRT1 interacts with Akt and associated binding proteins. To assess the mechanism of SIRT1 under hypoxia, a search was conducted for its partner proteins. Immunoprecipitation was performed to detect the co-expression of partner proteins. SIRT1 was immunoprecipitated from osteoblast lysates and partner proteins that were pulled down in the precipitate identified (Fig. 3). It was identified that Akt, a key protein in the apoptosis pathway, successfully interacted with SIRT1. The Akt protein pulled down with SIRT1 but not with the IgG negative control. To obtain further evidence for a SIRT1/Akt interaction, an inverse experiment was performed in which SIRT1 was intended to be pulled down by Akt. Lysine is normally an interaction residue for SIRT1, so it may also interact with Akt. To confirm this hypothesis, another immunoprecipitation was performed to demonstrate that Akt interacted with lysine; the resulting beads were analyzed by western blotting. These results demonstrated that Akt physically binds to SIRT1 and that lysine is capable of interacting with Akt.

SIRT1 interference of caspase expression eliminates hypoxia-induced apoptosis. During the apoptosis process, two signaling pathways are activated, the canonical and non-canonical. Previous studies have demonstrated that cytochrome *c*, apoptotic protease activating factor 1, and pro-caspase 9 released from the mitochondria interact with each other to form the apoptosome that drives the activation of caspase 3 (15,16). Due to the crucial role of caspases 3 and 9 in the apoptotic process, their protein expression was detected by western blotting. The hypoxia treatment potentiated apoptosis and the active forms of caspases 3 and 9 were increased. A high level of cleaved caspases 3 and 9 were detected. SIRT1 silencing treatment further increased the amount of cleaved caspases 3 or 9. In contrast, SIRT1 overexpression eliminated the expression of active forms of caspases under hypoxia (Fig. 4A). These findings suggest that SIRT1 may exert its regulatory effect on MC3T3-E1 cell apoptosis by regulating the ratio of pro-caspase 9/caspase 9 and pro-caspase 3/caspase 3.

SIRT1-activates protective signaling pathways in hypoxia-induced apoptosis. The Bcl-2 family regulates the canonical pathway of apoptosis by activation or suppression. Of the Bcl-2 family members, Bax and Bcl-2 are recognized as two of the most important, that exert either pro- or anti-apoptotic effects in cells (17). In response to hypoxic stress, Bax and Bcl-2 protein levels were altered by the differing amounts of SIRT1 present in the MC3T3-E1 cells (Fig. 4A). Bax was abundant in SIRT1 silenced MC3T3-E1 cells. In contrast, the Bax level was significantly reduced in SIRT1 overexpressed cells. Bcl-2, the anti-apoptosis protein, demonstrated the opposite phenotype and was markedly increased in SIRT1 overexpression cells. Several proteins or transcription factors were additionally detected using western blotting or RT-qPCR (Fig. 4B). Levels of Akt, p65, TRAF2, and cIAP2 were significantly lower in SIRT1 silenced cells compared with SIRT1 overexpressed cells. Together, these results suggested that SIRT1 attenuated the apoptosis of MC3T3-E1 cells by regulating the expression levels of various proteins.

Discussion

The present study to the best of our knowledge, presents the first evidence that SIRT1 modulates hypoxia-induced apoptosis in osteoblast cells through modulated caspase activation. To date, SIRT1 has been reported to interact with certain nuclear proteins, including p53 (18) or fork head family proteins (11,19), and modulate the functions of these proteins through deacetylation. The present study demonstrated that SIRT1 may deacetylate Akt and stimulate a protective effect through phosphatization, which reduces caspase

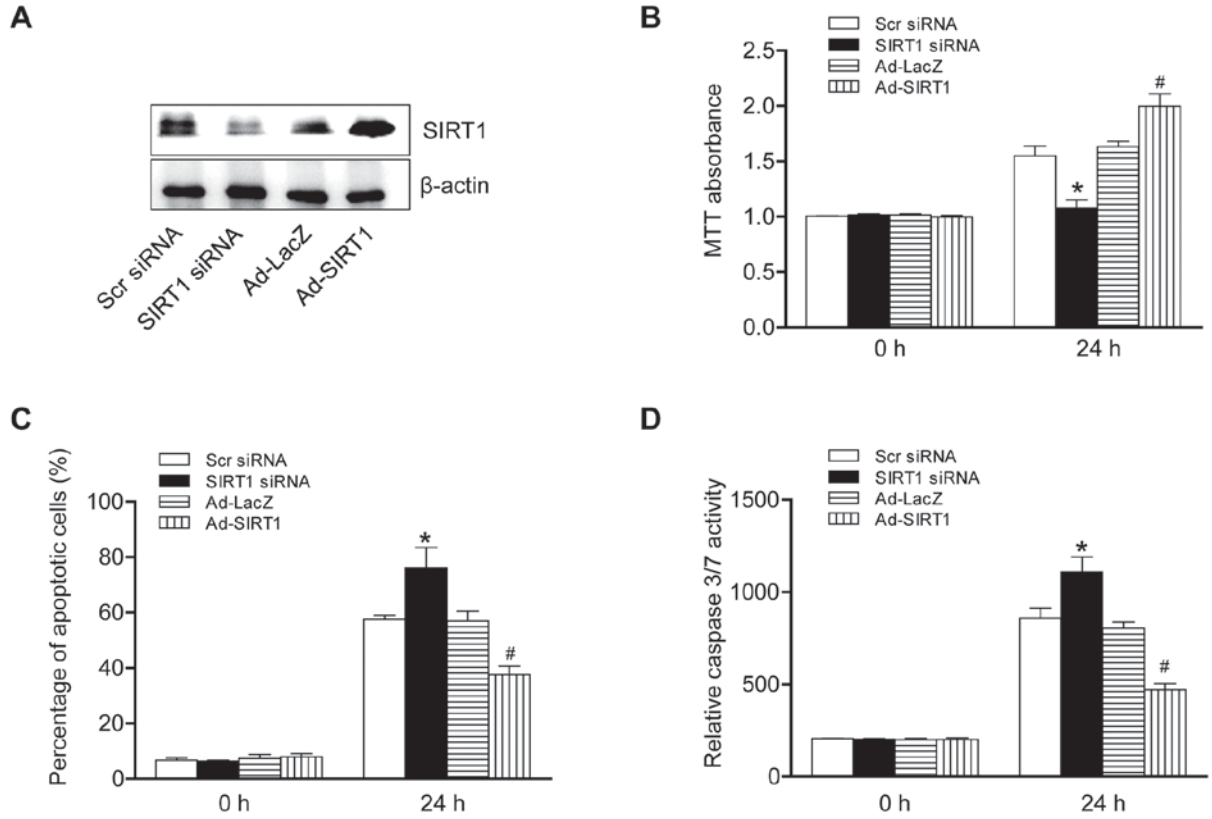

Figure 2. SIRT1 interference affects the development of hypoxia-induced apoptosis in MC3T3-E1 cells. MC3T3-E1 cells were treated with Ad-LacZ, Ad-SIRT1, scramble siRNA and SIRT1 siRNA, and then MC3T3-E1 cells were exposed to hypoxia for 24 h to induce cell apoptosis. (A) The protein expression level of SIRT1 was detected by western blotting. (B) Cell viability was determined using an MTT assay. (C) The apoptotic cells were analysed by POPercentage apoptosis assay kit. (D) The activity of caspases 3 and 7 were determined using commercially available assay kits. Results are expressed as the mean ± standard error of the mean of three independent experiments. Each sample was analyzed in triplicate. *P<0.05 vs. Scr siRNA group. #P<0.05 vs. Ad-LacZ group. The data are presented as the mean ± standard deviation of three independent experiments. SIRT1, nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1; siRNA, small interfering RNA; Scr, scrambled; Ad, adenovirus; Ad-Lacz, lacZ-bearing adenovirus.

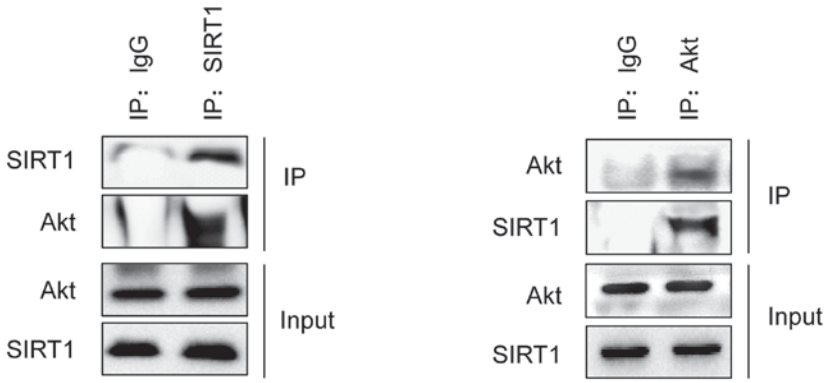

Figure 3. SIRT1 interacts with Akt. SIRT1 and Akt were immunoprecipitated in whole cell lysates and their interaction was observed. Whole cell lysates were immunoblotted with the indicated antibodies. SIRT1, nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1; IgG, immunoglobulin G; Akt, protein kinase B; IP, immunoprecipitated.

activation, and that SIRT1 inhibits hypoxia-induced apoptosis in osteoblasts.

The POPercentage apoptosis assay kit is an efficient assay for identifying cell apoptosis and providing direct quantification. Using this assay, it was observed that SIRT1 overexpressed cells exhibited decreased levels of apoptosis. The caspase 3 and 7 activity assay demonstrated that the canonical pathway of apoptosis was involved in SIRT1 signaling. Caspases are a family of protease enzymes serving essential roles in programmed cell death (20). Caspase 9 is an initiator caspase in the apoptosis pathway. The aspartic acid specific protease caspase 9 has been linked to the mitochondrial death pathway. It improves and activates the effector caspase, caspase 3, which is a major effector caspase responsible for the cleavage of cellular substrates during apoptosis (21). In the present study, it was identified that hypoxia treatment resulted in a significant increase in cleaved caspases 3 or 9. SIRT1 interference significantly altered the amount of the cleaved caspase and abrogated

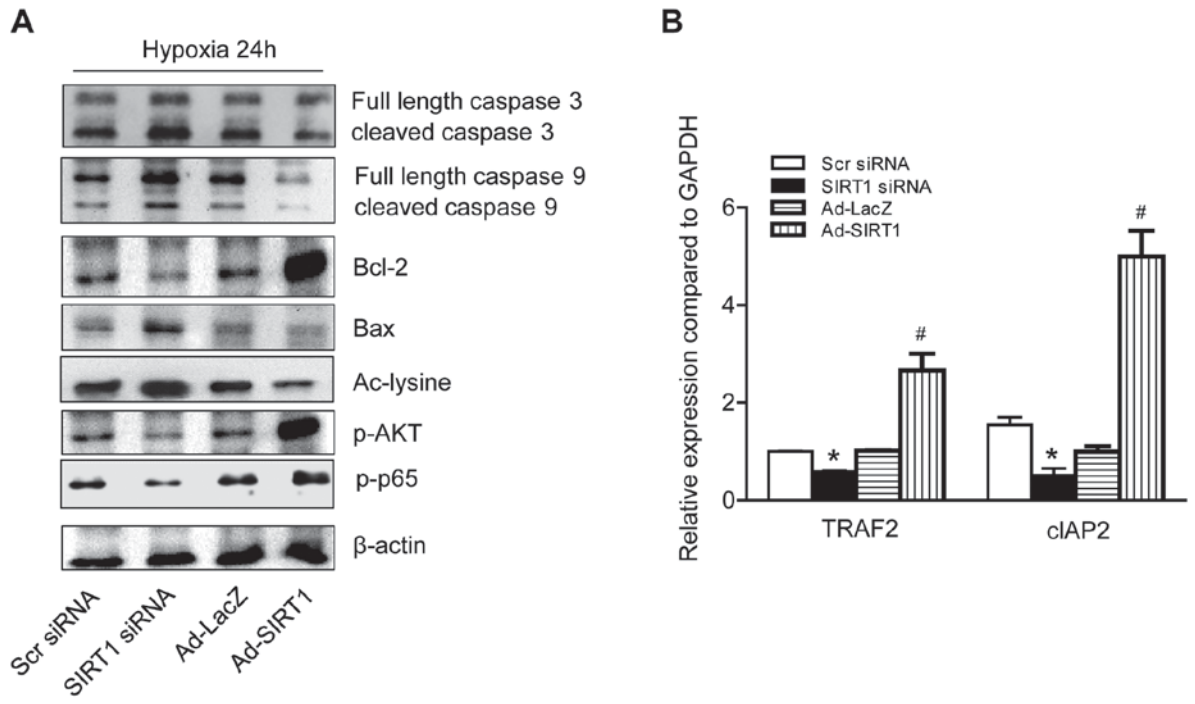

Figure 4. SIRT1 modifies apoptosis signaling pathway in hypoxic conditions. (A) MC3T3-E1 cells were transfected with SIRT1 and SIRT1 siRNA. Western blot analysis was performed to detect the total amount of Bax, Bcl-2, p-AKT, p-p65 and caspase 3 and 9 expression in cells. (B) MC3T3-E1 cells were treated as demonstrated in (A), and then were exposed to hypoxia for 24 h. The group without hypoxia treatment was taken as the control group. Reverse transcription-quantitative polymerase chain reaction was performed to detect the transfactors TRAF2 and cIAP2 in the hypoxia cells. *P<0.05 vs. Scr siRNA group. #P<0.05 vs. Ad-LacZ group. The data are presented as the mean ± standard deviation of three independent experiments. SIRT1, nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1; siRNA, small interfering RNA; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 associated X, apoptosis regulator; p, phosphorylated; Akt, protein kinase B; TRAF2, tumor necrosis factor receptor-associated factor 2; Scr, scrambled; Ad, adenovirus; Ad-Lacz, lacZ-bearing adenovirus.

caspase-mediated apoptosis. These results suggested that SIRT1 regulates the ratio of pro-caspase 9/caspase 9 and pro-caspase 3/caspase 3, which in turn may affect the development of MC3T3-E1 cell apoptosis.

SIRT1 is a NAD^+-dependent deacetylase and essential for cell survival, including the process of apoptosis. The lysine residues on proteins acetylate or deacetylate and may affect a number of protein functions, including transcriptional activity, DNA binding, protein binding, protein stability and translocation. Previously (22), SIRT1 has been reported to promote cell survival rate through mediating deacetylation of apoptosis-inducible nuclear proteins. The phosphorylation of p53, FOXO and Ku70 can inhibit proapoptotic signalling, as it decreases caspase 3/9 levels (22). Consistent with this finding, it was hypothesized that SIRT1-mediated phosphorylation of Akt or nuclear factor κB may also occur through the deacetylation of lysine residues, thus leading to protein activation and the induction of cell apoptosis. The western blotting data demonstrated that more Akt and p65 protein was phosphorylated in SIRT1 overexpressed cells. The activated proteins promoted transcription in the nucleus, and upregulated expression of protective genes, including TRAF2 and cIAPs (23).

These anti-apoptotic genes promote an alternation between Bax and Bcl-2. Bcl-2 family members form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. As two typical proteins of the Bcl family that restrain and promote apoptosis, Bcl-2 and Bax serve key roles in regulating the effect of mitochondrial membrane permeability, mitochondrial function and cytochrome *c* release; pro-apoptotic members induce apoptosis and anti-apoptotic members inhibit apoptosis (24). Therefore, the balance between pro- and anti-apoptotic members appears to be of critical importance. SIRT1 knockdown resulted in increased amounts of Bax, a pro-apoptotic member of the Bcl family. In contrast, the amount of Bcl-2, an anti-apoptotic member of the Bcl family, was markedly reduced. These observations suggest that increased Bax coupled with reduced Bcl-2 expression was, at least in part, one of the mechanisms resulting in increased apoptosis by the knockdown of SIRT1. The present study identified that SIRT1 may affect cell survival by mediating Bax and Bcl-2 expression levels. The hypoxia stimulus led to a significant increase of Bax. The amount of SIRT1 significantly affected the amount of Bax and Bcl-2 protein, suggesting that the Bcl family balance serves a critical role in the development of SIRT-mediated cellular apoptosis.

In summary, SIRT1 was identified as an important regulator of cellular apoptosis. Its expression was markedly reduced in hypoxia-induced apoptosis in MC3T3-E1 osteoblast cells. SIRT1 interference significantly alters MC3T3-E1 cell viability and affects the number of apoptotic cells and activity of caspases 3 and 7. Furthermore, SIRT1 expression may affect the activity of PI3K/AKT and NF-κB and alter the Bcl family balance, which in turn alters the development of MC3T3-E1 cell apoptosis. Therefore, SIRT1 acts as an important regulator of MC3T3-E1 cell apoptosis. SIRT1 intervention may potentially provide a novel strategy for the treatment of ischemic necrosis of the femoral head by controlling apoptosis.

Acknowledgements

The present study was supported by the National Research Foundation of Korea funded by the Korean government (grant no. NRF-2015R1A2A2A01003762), the Fund of Biomedical Research Institute of Chonbuk National University Hospital, and the Shandong Provincial Natural Science Foundation, China (grant no. ZR2014CQ037).

References

1. Bejar J, Peled E and Boss JH: Vasculature deprivation-induced osteonecrosis of the rat femoral head as a model for therapeutic trials. Theor Biol Med Model 2: 24, 2005.
2. Kerachian MA, Harvey EJ, Cournoyer D, Chow TY and Seguin C: Avascular necrosis of the femoral head: Vascular hypotheses. Endothelium 13: 237-244, 2006.
3. Pop C and Salvesen GS: Human caspases: Activation, specificity, and regulation. J Biol Chem 284: 21777-21781, 2009.
4. Verdin E, Hirschey MD, Finley LW and Haigis MC: Sirtuin regulation of mitochondria: Energy production, apoptosis, and signaling. Trends Biochem Sci 35: 669-675, 2010.
5. Imai S and Guarente L: Ten years of NAD-dependent SIR2 family deacetylases: Implications for metabolic diseases. Trends Pharmacol Sci 31: 212-220, 2010.
6. Baur JA, Ungvari Z, Minor RK, Le Couteur DG and de Cabo R: Are sirtuins viable targets for improving healthspan and lifespan? Nat Rev Drug Discov 11: 443-461, 2012.
7. Simic P, Zainabadi K, Bell E, Sykes DB, Saez B, Lotinun S, Baron R, Scadden D, Schipani E and Guarente L: SIRT1 regulates differentiation of mesenchymal stem cells by deacetylating β-catenin. EMBO Mol Med 5: 430-440, 2013.
8. Alcendor RR, Gao S, Zhai P, Zablocki D, Holle E, Yu X, Tian B, Wagner T, Vatner SF and Sadoshima J: Sirt1 regulates aging and resistance to oxidative stress in the heart. Circ Res 100: 1512-1521, 2007.
9. Takayama K, Ishida K, Matsushita T, Fujita N, Hayashi S, Sasaki K, Tei K, Kubo S, Matsumoto T, Fujioka H, *et al*: SIRT1 regulation of apoptosis of human chondrocytes. Arthritis Rheum 60: 2731-2740, 2009.
10. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L and Weinberg RA: hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 107: 149-159, 2001.
11. Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, McBurney M and Guarente L: Mammalian SIRT1 represses forkhead transcription factors. Cell 116: 551-563, 2004.
12. Lin CH, Lin CC, Ting WJ, Pai PY, Kuo CH, Ho TJ, Kuo WW, Chang CH, Huang CY and Lin WT: Resveratrol enhanced FOXO3 phosphorylation via synergetic activation of SIRT1 and PI3K/Akt signaling to improve the effects of exercise in elderly rat hearts. Age (Dordr) 36: 9705, 2014.
13. Wang J, Koh HW, Zhou L, Bae UJ, Lee HS, Bang IH, Ka SO, Oh SH, Bae EJ and Park BH: Sirtuin 2 aggravates postischemic liver injury by deacetylating mitogen-activated protein kinase phosphatase-1. Hepatology 65: 225-236, 2017.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
15. Fister S, Günthert AR, Aicher B, Paulini KW, Emons G and Gründker C: GnRH-II antagonists induce apoptosis in human endometrial, ovarian, and breast cancer cells via activation of stress-induced MAPKs p38 and JNK and proapoptotic protein Bax. Cancer Res 69: 6473-6481, 2009.
16. Yuan S, Yu X, Asara JM, Heuser JE, Ludtke SJ and Akey CW: The holo-apoptosome: Activation of procaspase-9 and interactions with caspase-3. Structure 19: 1084-1096, 2011.
17. Birkinshaw RW and Czabotar PE: The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. Semin Cell Dev Biol pii: S1084-S9521, 2017.
18. Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L and Gu W: Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 107: 137-148, 2001.
19. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, *et al*: Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 303: 2011-2015, 2004.
20. Chipuk JE and Green DR: How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends Cell Biol 18: 157-164, 2008.
21. Wang C and Youle RJ: The role of mitochondria in apoptosis*. Annu Rev Genet 43: 95-118, 2009.
22. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R and Sinclair DA: Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 305: 390-392, 2004.
23. Fu W, Liao X, Ruan J, Li X, Chen L, Wang B, Wang K and Zhou J: Recombinant human erythropoietin preconditioning attenuates liver ischemia reperfusion injury through the phosphatidylinositol-3 kinase/AKT/endothelial nitric oxide synthase pathway. J Surg Res 183: 876-884, 2013.
24. Leber B, Lin J and Andrews DW: Embedded together: The life and death consequences of interaction of the Bcl-2 family with membranes. Apoptosis 12: 897-911, 2007.