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STRESS-Responsive Deacetylase SIRT3 is Up-Regulated by Areca Nut Extract-Induced Oxidative Stress in Human Oral Keratinocytes

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ABSTRACT

Areca chewing is an important environmental risk factor for development of oral premalignant lesions and cancer. Epidemiological evidence indicates that areca chewing is tightly linked to oral carcinogenesis. However, the pathogenetic impacts of areca nut extract (ANE) on normal human oral keratinocytes (HOKs) are unclear and possibly involve oxidative stress via redox imbalance. Sirtuin 3 (SIRT3) is a member of the sirtuin family of proteins that play an important role in regulating cellular reactive oxygen species (ROS) production. Recent studies have confirmed that ANE and other areca ingredients can induce ROS. In this study, we examined the role of SIRT3 in the regulation of ANE-induced ROS in HOK cells. We examined HOK cell viability following treatment with various ANE concentrations. ANE-induced cytotoxicity increased in a dose-dependent manner and was approximately 48% at a concentration of 50 μg/ml after 24 h. SIRT3 expression and enzyme activity were up-regulated in HOK cells by ANE-induced oxidative stress. Additionally, we identified that SIRT3 controls the enzymatic activity of mitochondrial proteins, such as forkhead box O3a (Foxo3a) transcription factor and antioxidant-encoding gene superoxide dismutase 2 (SOD2), by deacetylation in HOK cells. Moreover, SIRT3-mediated deacetylation and activation of Foxo3a promotes nuclear localization *in vivo*. These findings suggest that SIRT3 is an endogenous negative regulator in response to ANE-induced oxidative stress and demonstrate an essential role for redox balance in HOK cells. J. Cell. Biochem. 115: 328–339, 2014.

KEY WORDS: ARECA NUT EXTRACT; SIRTUIN 3; REACTIVE OXYGEN SPECIES; HUMAN ORAL KERATINOCYTE

The gingiva is constantly being challenged by various stimuli within the oral environment. It is increasingly appreciated that epithelial tissues such as the gingival epithelia are not merely passive barriers to infection, but have important roles in inflammatory responses and oral neoplastic disease. There are approximately 600 million habitual betel quid chewers in South and Southeastern Asia, and various epidemiology studies have consistently indicated that betel quid chewing is related to a high incidence of oral premalignant lesions and cancers [Jeng et al., 2001; Sharma, 2003]. Among various areca ingredients include areca nut, limes, *Piper Betle* inflorescence, which contain polyphenols, arecoline, arecaidine, crude fiber and other compounds [Nair et al., 1992]. In 2004, areca nut components were evaluated as group I carcinogens in humans based on the potential carcinogenicity of betel quid [IARC, 2004]. Previous studies have revealed that areca nut extract (ANE) is cytotoxic and genotoxic to oral keratinocytes [Sundqvist et al., 1989], which is the main cell

Abbreviations used: HOK, human oral keratinocyte; ROS, reactive oxygen species; SIRT3, Sirtuin 3; ANE, areca nut extract; Foxo3a, forkhead box O3a; CAT, catalase; SOD2, superoxide dismutase 2; NAC, N-acetyl cysteine; NAM, nicotinamide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DHE, dihydroethidium; siRNA, small interfering RNA.

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type in gingival epithelial tissues. ANE and accompanying ingredients can induce oxidative stress, elicit reactive oxygen species (ROS) and activate stress responses in epithelial cells [Liu et al., 1996; Chang et al., 2001; Tang et al., 2003]. The impacts of ANE-induced ROS on oral keratinocytes are largely unclear, and the mechanism for protecting oral keratinocytes from oxidative stress through induction of several key ROS-detoxifying enzymes is unknown.

ROS can react with DNA, proteins, and lipids and play important roles in many physiological and pathophysiological conditions, such as diabetes, neurodegenerative diseases, cancer, and aging [Balaban et al., 2005]. Although ROS is produced in multiple cell compartments, the mitochondria are a major source of cellular ROS (approximately 90%) [Balaban et al., 2005]. ROS is generated primarily through the mitochondrial respiratory chain [Balaban et al., 2005] where electrons escape the electron transport chain and react with molecular oxygen, leading to the generation of superoxide.

Sirtuin (SIRT) proteins are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases and can also function as ADP-ribosyl transferases. Sirtuin proteins are involved in critical cellular processes, including oxidative stress responses, genomic stability, cell survival, development, metabolism, aging, and longevity of organisms ranging from yeasts to humans [Haigis and Guarente, 2006; Michan and Sinclair, 2007; Saunders and Verdin, 2007]. Of the seven SIRT analogues, SIRT3 is the only member whose increased expression has been associated with longevity, leading to an extended life span in humans [Rose et al., 2003; Bellizzi et al., 2005]. SIRT3 is a mitochondrial inner membrane protein [Onyango et al., 2002], which is synthesized as an inactive protein and subsequently activated by a matrix peptidase [Schwer et al., 2002]. This process allows SIRT3 to act on numerous substrates to activate fat oxidation, amino acid metabolism and electron transport [Huang et al., 2010]. SIRT3 was shown to regulate the activity of acetyl-CoA synthetase2, an important mitochondrial enzyme involved in generating acetyl-CoA for the tricarboxylic acid cycle [Hallows et al., 2006]. Forkhead transcription factor Foxo3a is also a substrate of SIRT3 [Jacobs et al., 2008; Sundaresan et al., 2009]. SIRT3 interacts with Foxo3a in the mitochondria and increases Foxo3a-dependent gene expression. SIRT3 blocks the cardiac hypertrophic response through activation of Foxo3a-dependent antioxidants in mice. SIRT3-deficient animals exhibit increased mitochondrial protein hyperacetylation, revealing SIRT3 as a major mitochondrial deacetylase [Lombard et al., 2007].

Recent studies have demonstrated a key role of SIRT3 as a mitochondrial-localized tumor suppressor [Kim et al., 2010; Bell et al., 2011; Finley et al., 2011]. The studies noted that SIRT3$^{-/-}$ mouse embryonic fibroblasts exhibited increased ROS [Kim et al., 2010] and that in vivo overexpression of *Sirt3* suppressed cellular ROS levels [Bell et al., 2011]. Although ROS levels were increased in SIRT3$^{-/-}$ cells, the cells also contain detoxification enzymes that should scavenge increased ROS. Thus, cells lacking *Sirt3* may have dysfunctional coordination of mitochondrial respiratory chain and detoxification enzymes, which can result in aberrant and potentially damaging ROS levels. This evidence suggests that SIRT3 could have a pivotal role in oxidative stress responses. The present study was designed to examine the role of SIRT3 in primary human oral keratinocytes (HOK). SIRT3 was observed to be expressed in keratinocytes and localized in both the mitochondria and the nucleus. We found that *Sirt3* gene expression, which is dependent on ROS pathway regulation, was significantly increased following ANE stimulation. By modulating SIRT3 levels in keratinocytes using either overexpression or knockdown, we showed that SIRT3 protects oral keratinocytes by suppressing oxidative stress. In this study, we demonstrate that knockdown SIRT3 increased HOK cell growth and proliferation, indicating that SIRT3 has a role in maintenance of normal cells. Moreover, ANE stimulation of HOK cells led to ROS genesis and SIRT3 up-regulation, but did not have an effect on knockdown cells, demonstrating a role for SIRT3 as an oral keratinocyte regulator under oxidative stress conditions.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

HOK cells were cultured in oral keratinocyte growth medium (ScienCell, Carlsbad, CA) in a 37°C incubator with 5% CO_2 and were passaged routinely at 90% confluence. ANE was purchased from a local commercial store and homogenized with solution. Aqueous ANE was immediately passed through 6-μm (Advantec, Tokyo, Japan) and 0.22-μm (Millipore, Bedford, MA) filters and stored freeze-dried at −20°C until use.

CELL VIABILITY ASSAY

For dose-response experiments, 10^4 HOK cells were refreshed and cultured for 24 h in a 96-well plate. To assess cell viability, cells were treated with various concentrations (0, 10, 25, 50, 100, and 200 μg/ml) of ANE for 24 h, and cell activity was quantified via mitochondrial dehydrogenase using an in vitro toxicology assay kit (MTS-based, Promega, Madison, WI). Absorbance at 490 and 690 nm was measured using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA), and quantification of average cell viability (%) was performed in triplicate for each treatment.

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen, Camarillo, CA). cDNA was reverse-transcribed and amplified by PCR using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). Quantitative RT-PCR was performed using the FastStart Universal SYBR Green Master (Roche) on an Applied Biosystems ABI 7900 RealTime PCR System (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following sets of primers were used for amplification: SIRT3-F 5′-ACCCAGTGGCATTCCAGAC-3′, SIRT3-R 5′-GGCTTGGGGTTGTGAAAGAAG-3′; SOD2-F 5′-ACAGGCCTTATTCCACTGCT-3′, SOD2-R 5′-CAGCATAACGATCGTGGTTT-3′; CAT-F 5′-TAAGACTGACCAGGGCATC-3′, CAT-R 5′-CAAACCTTGGTGAGATCGAA-3′; Foxo3a-F 5′-TGCTAAGCAGGCCTCATCTC-3′, Foxo3a-R 5′-GCTCCCTGGACACCCATTCC-3′; GADD45-F 5′-AAACAGAAACGGAAGAAGGAGGCG-3′, GADD45-R 5′-ACTGCCAGGTAGCAGGCTTTATTG-3′; and GAPDH-F 5′-GAGTCAACGGATTTGGTCGT-3′, GAPDH-R 5′-GACAAGCTTCCCGTTCTCAG-3′. Gene expression levels were normalized using GAPDH as an internal reference gene, and the average relative change was

calculated using determinations by relative quantification, applying the delta-delta cycle threshold method.

TRANSIENT TRANSFECTION

HOK cells were transiently transfected with small interfering RNA (siRNA) (150 nM; a pool of three target-specific siRNA) against SIRT3 or with a nontargeting control (Santa Cruz Biotechnology, Santa Cruz, CA) in serum free medium containing Lipofectamine Plus (Invitrogen). Transfection efficiency was assessed by Western blot.

CYTOSOLIC, NUCLEAR, MITOCHONDRIA ISOLATION AND IMMUNOPRECIPITATION

Cytosolic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, Basingstoke, UK). Mitochondria were isolated from cultured cells using a mitochondria isolation kit (Pierce Biotechnology, Inc., Rockford, IL) following the manufacturer's protocol. Isolated mitochondria were lysed with RIPA buffer and analyzed by direct Western blot or immunoprecipitation. Two milligrams of protein from samples (total lysates or mitochondrial extracts) was used for immunoprecipitation with a Pierce® Crosslink IP kit (Pierce) following the manufacturer's protocol and analyzed by Western blot.

WESTERN BLOT ANALYSIS

Cells were lysed directly in RIPA buffer and adjusted for protein concentration using the BCA protein assay kit (Bio-Rad, Hercules, CA). Lysates were resolved by 10% SDS–PAGE and transferred to PVDF membranes. The membranes were blocked and incubated with specific antibodies against SIRT3 (GeneTex), actin (Sigma–Aldrich, St. Louis, MO), GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), SOD2 (Epitomics, San Diego, CA), LCAD (Pierce), and acetylated-lysine (Cell Signaling). Proteins were visualized by enhanced chemiluminescence using an ECL-Plus detection system (Perkin Elmer-NEN, Courtaboeuf, France).

ROS MEASUREMENT

Cellular ROS was detected using the fluorescent dye dihydroethidium (DHE) obtained from Vigorous (Vigorous, Beijing, China) according to a previous study [Slane et al., 2006]. HOK cells were cultured in 6-well plates for 24 h and treated with or without ANE. The cells were then washed with PBS and labeled with DHE (5 μmol/L dissolved in 1% DMSO) in culture plates at 37°C for 30 min in PBS. Culture plates were placed on ice to stop the labeling process, trypsinized, and resuspended in ice-cold PBS. Samples were analyzed using a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA). The mean fluorescence intensity (MFI) of 10,000 cells was analyzed for each sample and corrected for auto-fluorescence from unlabeled cells.

ENZYME ACTIVITY ASSAY

The enzymatic activities of superoxide dismutase 2 and catalase were measured using a kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol. SIRT3 proteins from total lysates of cultured cells were concentrated using a Pierce® Crosslink IP kit (Pierce), according to the manufacturer's recommendations. Protein concentration was determined using a protein assay kit (Bio-Rad). A SIRT3 enzyme activity assay was performed in 50 μl of deacetylase buffer (4 mM, $MgCl_2$, 0.2 mM dithiothreitol, 50 mM Tris–HCl, pH 8.5) containing 25 μl SIRT3 protein (10 ng/μl), 2 mM NAD^+, and 25 μl 1 mM fluorogenic peptide substrate Ac-Arg-Gly-Lys(Ac)-AMC (R&D systems, Minneapolis, MN). Deacetylation reactions were conducted at 37°C for 30 min and stopped by adding 50 μl of a stop solution consisting of recombinant mouse trypsin 3/PRSS3 AMC (R&D systems) and nicotinamide (Sigma–Aldrich) at final concentrations of 0.2 ng/μl and 4 mM, respectively. The assays were then incubated at room temperature for 15 min and read at excitation and emission wavelengths of 380 and 460 nm, respectively, in endpoint mode. Activity was measured with a SpectraMax M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

STATISTICAL ANALYSIS

Data are reported as the mean ± SD of at least three independent experiments. *P*-values for linear trends regarding mRNA expression levels were analyzed using the *t* test (slope estimate) in simple linear regression models. The difference was considered statistically significant at the level of $P < 0.05$ or $P < 0.01$.

RESULTS

CELL VIABILITY

Although the effects of ANE on oral keratinocyte growth in early passages (≤3rd passage) have been previously shown [Lu et al., 2006], differences could be due to the fact that ANE was purchased from a different source and use of various ANE preparation protocols from different areca species. In this study, we used a mitochondrial dehydrogenase assay to measure cell cytotoxicity following exposure to various concentrations of ANE ranging from 0 to 200 μg/ml for 24 and 48 h to investigate the effects of ANE on cultured HOK cells. As shown in Figure 1, ANE-induced cytotoxicity increased in a dose-dependent manner ($P < 0.05$) and was approximately 48% at a concentration of 50 μg/ml, although there was only a marginal

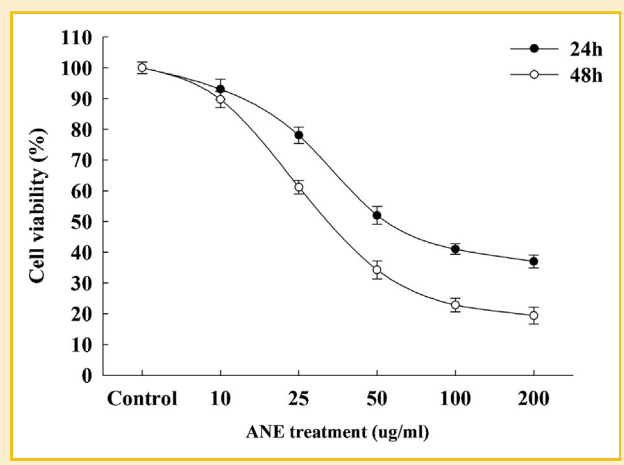

Fig. 1. Proliferation of HOK cells is inhibited by ANE. HOK cells (10^4) were incubated with various concentrations of ANE (control, 10, 25, 50, 100, and 200 μg/ml) for 24 or 48 h. Cell viability was determined by the MTS assay. Each data point represents the mean ± SD from at least three independent experiments.

increase in cytotoxicity when the concentration was increased to 200 μg/ml. HOK exposure to 25 and 50 μg/ml ANE for 48 h resulted in decreased cell proliferation by 39% and 66%, respectively. Therefore, treatment with 50 μg/ml ANE, which led to cytoinhibition, was used to evaluate the impact of ANE on HOK cells.

SIRT3 EXPRESSION IS ELEVATED DURING ANE STIMULATION

ANE is an inducer of oxidative stress and cellular ROS [Liu et al., 1996; Chang et al., 2001; Tang et al., 2003]. After treatment with 50 μg/ml ANE, ROS levels were significantly increased at different time points (Fig. 2A). ANE-induced increases in ROS levels were eliminated by pre-incubation with N-acetyl cysteine (NAC; Fig. 2B). Because SIRT3 is a stress-responsive deacetylase, we examined whether SIRT3 expression changed after an increase in ROS and measured SIRT3 protein levels after ANE stimulation in HOK cells. After exposure to 50 μg/ml ANE, total RNA was extracted from cells and SIRT3 mRNA was measured by real-time PCR. We observed that SIRT3 mRNA levels were increased by approximately 2.4- and 4.6-fold after 6 and 24 h of ANE stimulation in HOK cells, respectively. These increases in SIRT3 mRNA were accompanied by potent up-regulation of SIRT3 protein levels in HOK cells (Fig. 3A).

We evaluated whether ANE stimulation affected SIRT3 enzyme activity. After treating HOK cells with 50 μg/ml ANE for different lengths of time, SIRT3 enzyme activity was increased by approximately 35% within 2 h compared with controls (Fig. 3B). To further determine whether SIRT3 activity was increased by ANE-induced ROS, we used siRNA oligonucleotides to specifically knockdown SIRT3 gene expression. As shown in Figure 3C, SIRT3 expression levels were increased in both normal and siSIRT3 HOK cells, but the SIRT3 knockdown compromised the ability of ANE to activate the enzyme activity of SIRT3, which was decreased about threefold relative to control cells after ANE stimulation. In addition, the ANE-induced increase in ROS level was eliminated by pre-incubation with 10 mM NAC, and addition of NAC to HOK cells slightly reversed the increase in SIRT3 activity ($P < 0.05$). Moreover, siRNA against SIRT3 reduced ANE induction of endogenous SIRT3 protein levels in HOK cells. We observed that ANE stimulation and NAC pre-incubation did not change the enzyme activity of SIRT3 after knocking down SIRT3 gene expression. These results clearly indicate that ANE induces ROS generation and mediates ROS to stimulate SIRT3 expression and enzyme activity.

SIRT3 LOSS OF FUNCTION INCREASES PROLIFERATION AND CELLULAR ROS

Previous studies have suggested that ROS is directly associated with senescence and apoptosis in primary cells [Kim et al., 2010]. We aimed to determine the effect of SIRT3 deletion on cell proliferation of primary human oral keratinocytes by utilizing siRNA oligonucleotides against SIRT3 to suppress SIRT3 expression. siSIRT3 decreased basal SIRT3 protein levels by more than 70% compared with scrambled controls (Fig. 4A). We knocked down SIRT3 in HOK cells with siRNA oligonucleotides and found that knockdown cells displayed increased proliferation (Fig. 4A). To determine whether deleting SIRT3 resulted in increasing ROS levels in HOK cells, we measured intracellular ROS levels in normal and knockdown HOK cells treated with and without ANE. As shown in Figure 4B, basal superoxide levels were increased in HOK cells by transient transfection with siRNA compared to control cells, as evaluated with the ROS-activated fluorescent dye DHE. Moreover, the ROS level was significantly increased about 5.8-fold in siSIRT3 HOK cells relative to control cells after ANE stimulation. In contrast, when normal and knockdown cells were treated with nicotinamide (NAM), an inhibitor of most known deacetylases, there was an approximately 37% increase in ROS levels in normal cells compared with controls. After ANE stimulation, the ROS levels in normal and siSIRT3 HOK cells were increased by approximately 4.5- and 5.8-fold, respectively. In addition, NAM treatment further increased ROS levels in HOK cells about 6.4-fold relative to controls. However, NAM treatment did not further increase ROS levels in SIRT3 knockdown cells after ANE stimulation (Fig. 4C). These data demonstrate that deleting SIRT3 results in higher growth rates in HOK cells and that SIRT3 might play an important role in ROS production.

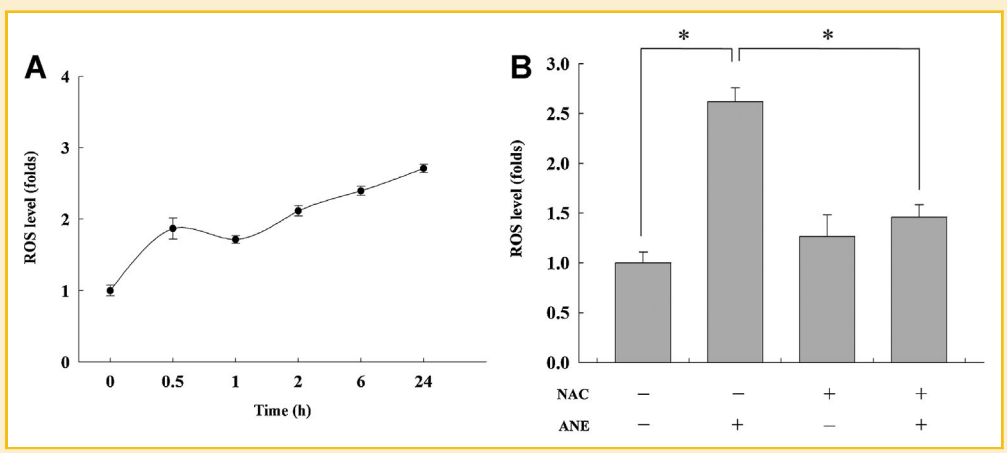

Fig. 2. ROS levels are induced by ANE in HOK cells. A: ROS levels in HOK cells were measured after treatment with or without 50 μg/ml ANE for 0–24 h. B: Abolishment of ROS by NAC pretreatment. ROS induced by ANE at 24 h was eliminated by pretreatment with 10 mM NAC for 1 h. Data are represented as the mean ± SD from at least three independent experiments. The asterisk indicates a significant difference (*, $P < 0.05$) compared to the control.

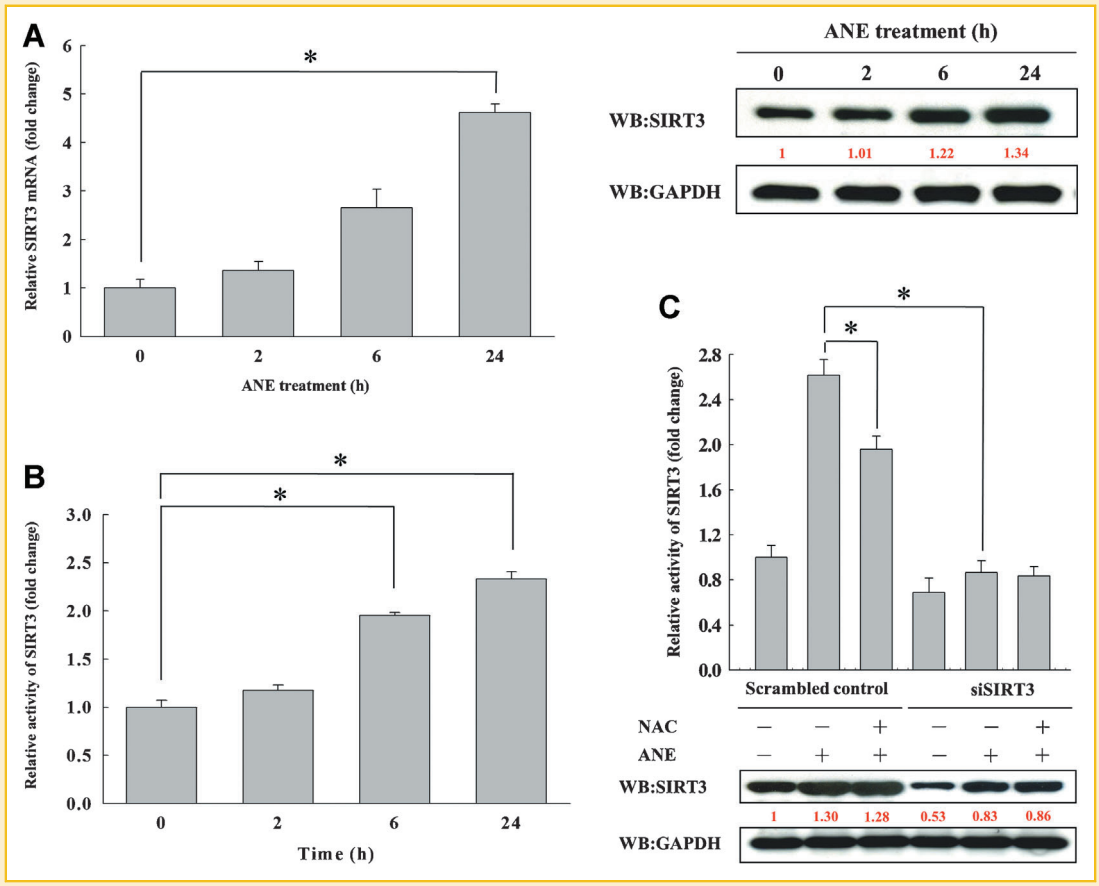

Fig. 3. SIRT3 levels are elevated by ANE in HOK cells. A: RT-PCR and Western blotting reveal SIRT3 expression in HOK cells after ANE stimulation. B: Specific activities of SIRT3 in HOK cells were determined by enzyme assay after ANE stimulation for 0–24 h. C: Relative activities of SIRT3 in HOK cells with or without siSIRT3 after ANE stimulation for 24 h. Protein levels were normalized to GAPDH. Quantification values are shown below the illustration. Data are represented as the mean ± SD from at least three independent experiments. The asterisk indicates a significant difference (*, $P < 0.05$) compared to the control.

SIRT3 KNOCKDOWN SUPPRESSES ANTIOXIDANT ENZYMES

Because loss of SIRT3 augments intracellular ROS levels, we investigated whether SIRT3 loss of function regulates antioxidant enzymes in HOK cells. As shown in Figure 5A, deleting SIRT3 led to a clear decrease in the gene expression of ROS-detoxifying enzymes, catalase (CAT) and superoxide dismutase 2 (SOD2). SOD2 and CAT protein levels were measured in normal and knockdown HOK cells and a decrease by approximately 19–26% and 5–23%, respectively, was observed in siSIRT3 HOK cells. These data demonstrate that SIRT3 functions as an upstream regulator of antioxidant genes.

SIRT3 MEDIATES THE ACTIVITY OF SOD2

SIRT3 is localized in the inner mitochondrial membrane [Onyango et al., 2002] along with electron transport chain proteins. Mitochondria are the major sites of superoxide formation, and accumulation of superoxide is believed to contribute to oxidative damage. Previous studies have suggested that ROS levels are increased in SIRT3$^{-/-}$ cells [Kim et al., 2010]. The authors found that glycolytic activity was increased, whereas mitochondrial ATP levels and maximal capacity of complexes I and III were reduced. ROS-mediated damage to mitochondrial DNA and loss of mitochondrial protein deacetylation in SIRT3$^{-/-}$ cells might explain these changes. Since ANE stimulation can increase SIRT3 expression levels and enzyme activity and knockdown of SIRT3 suppressed gene expression of ROS-detoxifying enzymes, it seemed logical to propose that SIRT3 mediates SOD2 deacetylation and activation. To test this hypothesis and determine the functional significance of SOD2-SIRT3 interaction, we immunopurified endogenous SOD2 and performed an activity assay. We found that SOD2 purified from HOK cells treated with NAM showed an approximately 40% decrease in specific activity compared with controls, whereas acetylation of SOD2 was increased (Fig. 5B). We immunopurified endogenous SOD2 from SIRT3-knockdown HOK cells treated with or without NAM and determined the specific activity of SOD2 and found that the absence of SIRT3 significantly increased the level of acetylated SOD2. In additional, NAM treatment reduced SOD2-specific activity by 46%, but had almost no effect on SOD2 activity in SIRT3 knockdown cells (Fig. 5B). This reduction in SOD2 activity by SIRT3 knockdown was similar to results of NAM treatment, which reduced SOD2 activity by 40%. Moreover, NAM treatment could not further inhibit SOD2 specific activity and

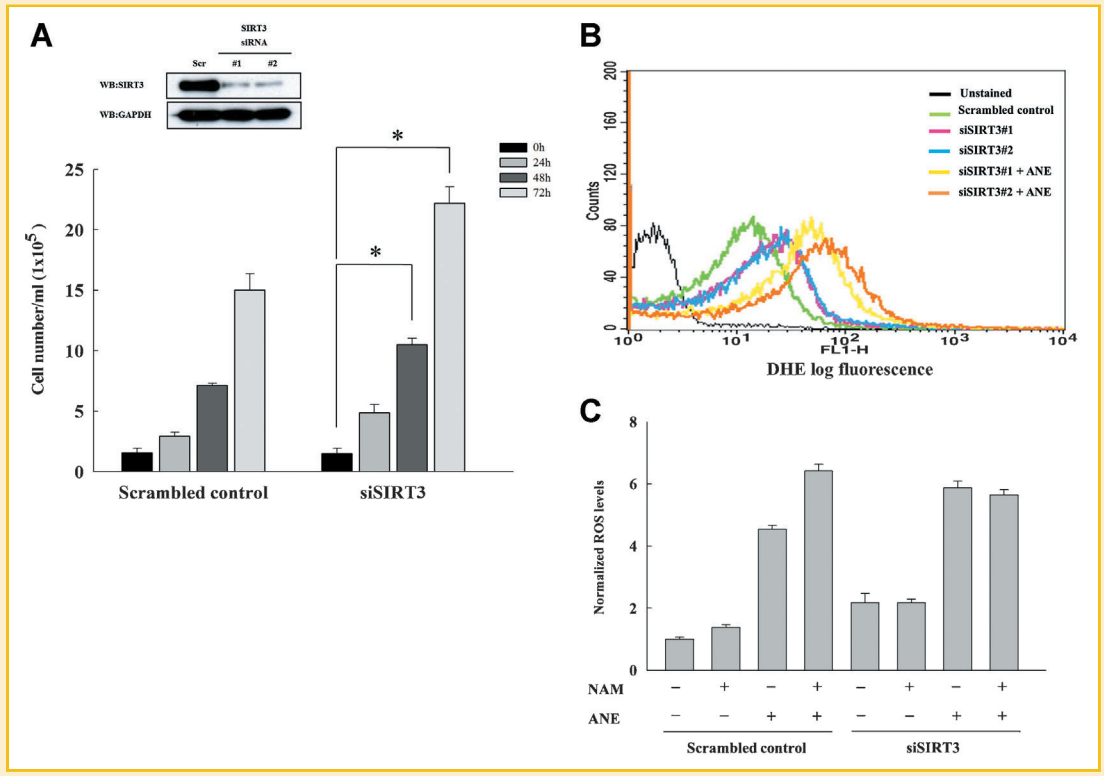

Fig. 4. SIRT3 loss of function increases proliferation and cellular ROS. A: Loss of SIRT3 increases cell proliferation in HOK cells. B: Relative levels of DHE fluorescence in scrambled controls and knockdown (siSIRT3) cells treated with 10 μM DHE for 30 min after ANE stimulation for 24 h and flow cytometry analysis using CellQuest software. C: Inhibition of deacetylases increases ROS levels in scrambled control cells treated with or without 10 mM NAM after ANE stimulation for 24 h, but NAM treatment only slightly increased ROS levels in the knockdown (siSIRT3) cells. Data are represented as the mean $\pm$ SD from at least three independent experiments. The asterisk shows a significant difference (*, $P < 0.05$) compared to the control.

increase acetylation of SOD2 in SIRT3 knockdown cells. Together, these results indicate that SIRT3 is the main deacetylase that activates SOD2 *in vivo* and has an effect on SOD2 activity in the ROS defense system.

ANE-INDUCED ROS STIMULATES SIRT3 EXPRESSION TO ACTIVATE SOD2

Because SIRT3 can promote SOD2 activity, we examined whether SOD2 deacetylation and enzyme activity changed after ANE stimulation in HOK cells. After exposure to 50 μg/ml ANE for different lengths of time, endogenous SOD2 in HOK cells was immunoprecipitated using a SIRT3 antibody and analyzed by Western blotting with SOD2 and anti-acetylated-lysine antibodies. SOD2 enzyme activity was increased 1.9- and 2.4-fold after 6 and 24 h of ANE treatment, respectively. In conjunction with an increase in SOD2 activity, SOD2 acetylation levels decreased following ANE treatment and were barely detectable after 6 h. However, there was almost no effect on SOD2 protein levels. Consistently, SIRT3 protein levels increased (Fig. 6A). To determine the enzyme activity and acetylation levels of SOD2 in siSIRT3 HOK cells after ANE stimulation at 50 μg/ml ANE for 24 h, we knocked down SIRT3 in HOK cells with siRNA oligonucleotides. Endogenous SOD2 in normal and siSIRT3 HOK cells was immunoprecipitated using a SIRT3 antibody and analyzed by Western blotting with SOD2 and anti-acetylated-lysine antibodies. SOD2 enzyme activity was increased 1.6-fold and acetylation of SOD2 was decreased 24% compared with controls after ANE treatment. Moreover, siSIRT3 HOK cells showed a significant increase in SOD2 acetylation with an associated decrease in SOD2 enzyme activity. Enzyme activity of SOD2 was decreased 0.5-fold in siSIRT3 HOK cells compared with normal cells. ANE stimulation caused deacetylation and activation of SOD2 in normal HOK cells, but had little effect on either acetylation levels or activities of siSIRT3 in treated and untreated HOK cells (Fig. 6B). These results suggest that ANE stimulation could induce *Sirt3* gene expression and protein levels, leading to increased deacetylation and activation of SOD2.

SIRT3 DEACETYLATES AND ACTIVATES THE FOXO3A TRANSCRIPTION FACTOR

Transcription factors belonging to the Foxo subfamily have been shown to inhibit ROS generation by enhancing the activity of SOD2 [Daitoku et al., 2004; Tan et al., 2008]. Because SIRT3 has been found to have redundant effects protecting cardiomyocytes during stress, we postulated that SIRT3 might be able to control the activity of Foxo3a in oral keratinocytes [Sundaresan et al., 2009]. To test this hypothesis, we first examined the ability of SIRT3 to bind to Foxo3a.

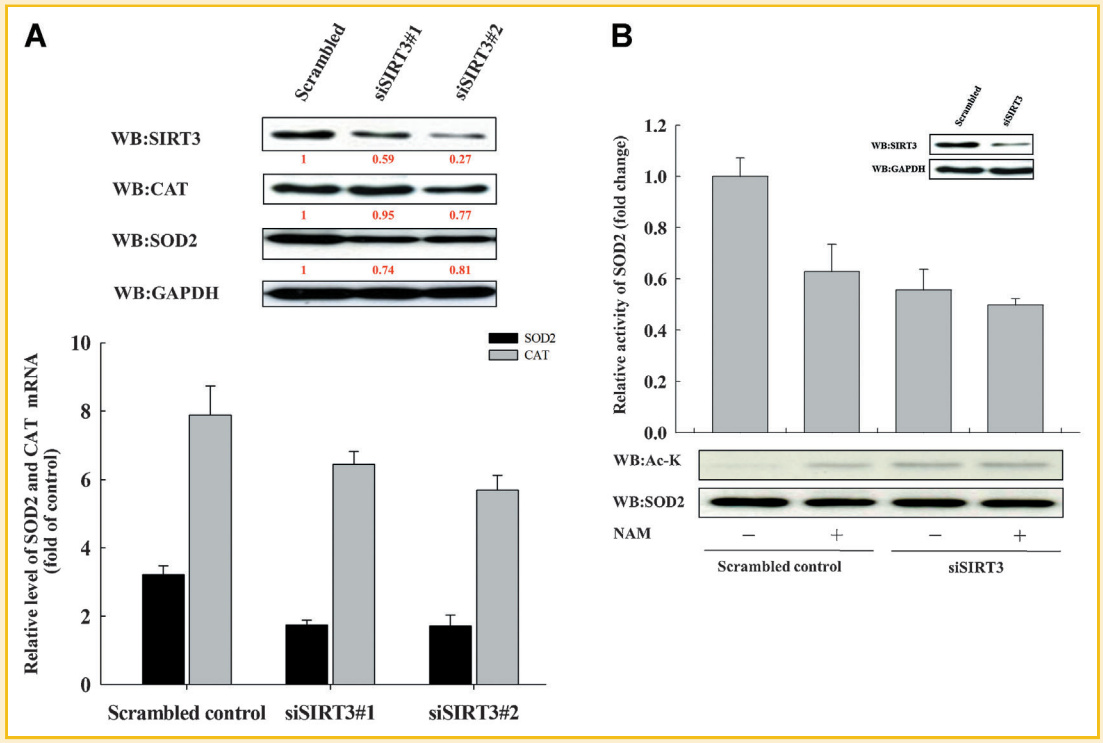

Fig. 5. SIRT3 mediates antioxidant enzyme expression and activity. A: Loss of SIRT3 increases antioxidant gene expression. RT-PCR and Western blotting reveal SOD2 and CAT expression in HOK cells with or without siSIRT3. B: Loss of SIRT3 increases endogenous SOD2 acetylation and decreases SOD2 activity. Acetylation of endogenous SOD2 in HOK cells with or without siSIRT3 was determined by immunopurification and probing by an acetyl lysine antibody. Protein levels were normalized to GAPDH. Quantification values are shown below the illustration. Data are represented as the mean ± SD from at least three independent experiments.

As shown in Figure 7A, SIRT3 and Foxo3a were able to bind to each other *in vivo*. To determine whether SIRT3 can directly lead to deacetylation of Foxo3a, siSIRT3 cells were treated with 10 μM H_2O_2 to induce acetylation of Foxo3a. We immunopurified endogenous Foxo3a from SIRT3 knockdown HOK cells and observed that the SIRT3 knockdown significantly increased the level of acetylated Foxo3a (Fig. 7B), suggesting that SIRT3 is capable of deacetylation of Foxo3a.

Nucleo-cytoplasmic shuttling controls the transcriptional activity of Foxo factors. Previous studies have shown that SIRT3 blocks phosphorylation-dependent nuclear export of the protein in cardiomyocytes [Sundaresan et al., 2009]. In this study, we evaluated whether SIRT3 could block phosphorylation-dependent nuclear export of the Foxo3a protein in oral keratinocytes by separating nuclear and cytoplasmic fractions from normal and knockdown HOK cells. Analysis of these fractions revealed that siSIRT3 cells decreased nuclear accumulation of Foxo3a compared with normal cells (Fig. 7C). Foxo3a was localized in the nucleus and cytoplasm of normal cells, whereas it was completely localized in the cytoplasm of siSIRT3 cells. Foxo3a was slightly localized in the nucleus after knocking down SIRT3 gene expression, which may result from decreased, but not completely eliminated, SIRT3 expression by siRNA. These results indicate that SIRT3-mediated deacetylation and activation of Foxo3a promotes its nuclear localization *in vivo*.

DEACETYLATION AND ACTIVATION OF FOXO3A BY SIRT3 FOLLOWING ANE STIMULATION

Earlier experiments showed that ANE-induced ROS stimulated SIRT3 expression and that SIRT3 could block phosphorylation-dependent nuclear export of Foxo3a protein in HOKs. Therefore, we examined whether the expression and enzyme activity of Foxo3a changed after ANE stimulation in HOK cells. In this study, we measured Foxo3a mRNA and protein levels after ANE treatment. After exposure to 50 μg/ml ANE, total RNA and protein were extracted from cells and measured by real-time PCR and Western blot analysis, respectively. We observed that Foxo3a mRNA and protein levels were nearly unchanged after 24 h of ANE stimulation in HOK cells (Fig. 8A). In addition, we measured acetylation of Foxo3a in HOK cells after 24 h with ANE or H_2O_2 (positive control). We immunopurified endogenous Foxo3a and found that Foxo3a purified from HOK cells treated with ANE or H_2O_2 increased SIRT3 protein levels and inhibited acetylation of Foxo3a (Fig. 8B). In conjunction with decreased Foxo3a acetylation, SIRT3 protein expression was increased after 24 h of ANE treatment, but Foxo3a protein levels were not. We separated and analyzed nuclear and cytoplasmic fractions from HOK cells treated with either ANE or H_2O_2 and found that treated cells had significantly increased nuclear accumulation of Foxo3a that was not observed in untreated HOK cells (Fig. 8C). While Foxo3a was localized in the nucleus and cytoplasm in normal HOK cells, it was

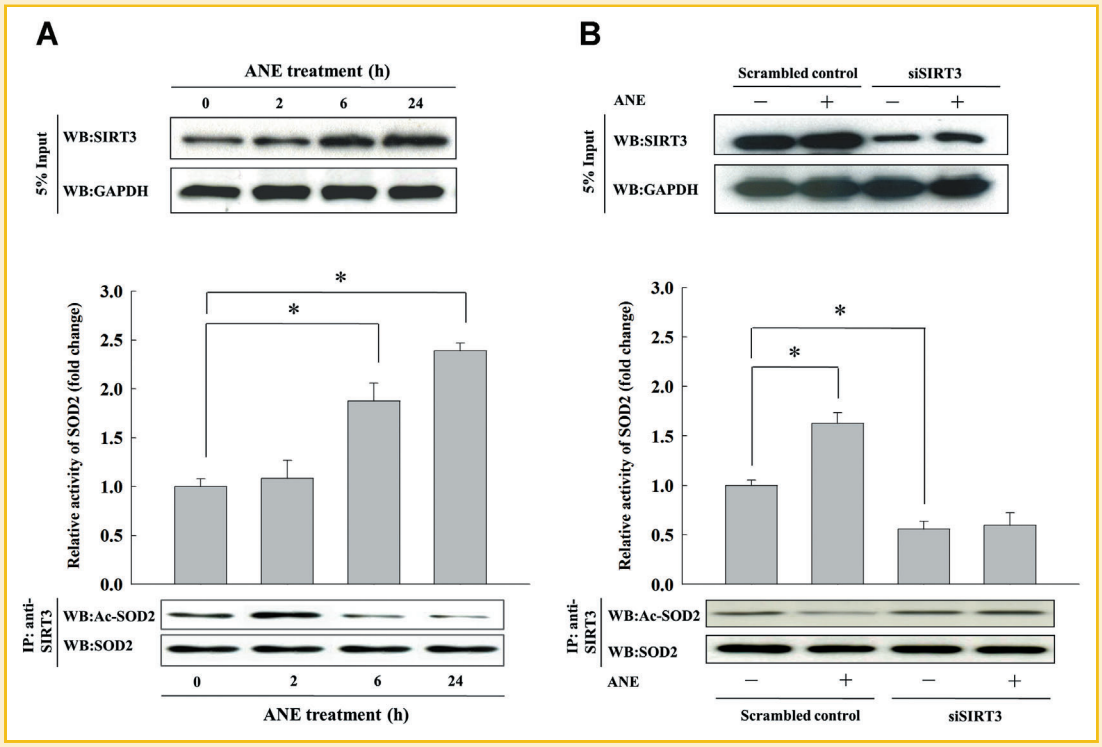

Fig. 6. ANE-induced ROS stimulates SIRT3 expression to activate SOD2. A: ANE stimulation decreases SOD2 acetylation and activates SOD2. Acetylation levels and specific activities of SOD2 expressed in HOK cells were determined after 50 μg/ml ANE treatment for 0–24 h. B: ANE regulates SOD2 through SIRT3. Acetylation levels and relative activities of SOD2 expressed in HOK cells with or without siSIRT3 treated with or without ANE. Acetylation levels of endogenous SOD2 in HOK cells with or without siSIRT3 were determined by immunopurification and probing by an acetyl lysine antibody. Data are represented as the mean ± SD from at least three independent experiments. The asterisk shows a significant difference (*, $P < 0.05$) compared to the control.

primarily localized in the nucleus of treated cells. SIRT3 expression of a ~44 kDa long and ~28 kDa short fragment was measured in untreated and treated HOK cells by Western blotting, and both forms of SIRT3 were increased in the treated HOK cells. The long SIRT3 fragment was localized preferentially in the cytoplasm of untreated and treated HOK cells, but the short fragment was only observed in the cytoplasmic fraction, which may be due to localization of the long form in the mitochondria, nucleus and cytoplasm and localization of the short form exclusively in the mitochondria. SIRT3 levels are increased in the cytoplasm and nucleus during stress [Schwer et al., 2002; Scher et al., 2007]. For additional evidence that ANE stimulation activated Foxo3a transcription activity by SIRT3, HOK cells were transiently transfected with siRNA oligonucleotides against SIRT3. mRNA levels of the Foxo3a-dependent target genes SOD2 and GADD45 [Tran et al., 2002a] were measured by real-time PCR (Fig. 8D). The SIRT3 knockdown compromised the ability of ANE to activate the transcriptional activity of Foxo3a, and gene expression of SOD2 and GADD45 was decreased about 4-fold and 2.1-fold, respectively, compared with control cells after ANE stimulation. These results suggest that ANE stimulation can induce SIRT3 protein expression, leading to increased deacetylation and nucleo-cytoplasmic shuttling of Foxo3a. Moreover, siRNA against SIRT3 reduced ANE induction of Foxo3a activation in HOK cells.

DISCUSSION

Keratinocytes are the main cell type in the epidermis with a primary function of forming a barrier against environmental damage. In the oral environment, the gingival epithelia are not merely passive barriers to infection, but have important roles in inflammatory responses and oral neoplastic disease [Marsh, 2003]. Damage to these cells by ANE might lead to oral pathogenesis. ANE exposure is known to be strongly associated with the habit of betel chewing, and it has been seriously considered the initial risk factor for oral premalignant lesions and oral cancers in betel chewers [Jeng et al., 2001; Sharma, 2003]. Previous studies specified that oxidative DNA damage in oral keratinocytes resulting from ANE exposure was an oxidative stress inducer [Liu et al., 1996; Chen et al., 2002], and other evidence indicates that ANE is genotoxic and cytotoxic to oral keratinocytes [Sundqvist et al., 1989; Liu et al., 1996; Chang et al., 2001; Lin et al., 2002]. To elucidate possible damage to HOK cells resulting from ANE exposure, we analyzed the impact of ANE on HOK cell growth using the 3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. ANE-induced cytotoxicity increased in a dose-dependent manner, revealing that a lower dose of ANE might stimulate the cells. However, a higher concentration of ANE may have increased the cytotoxic effects in our study. We identified that ANE could mediate

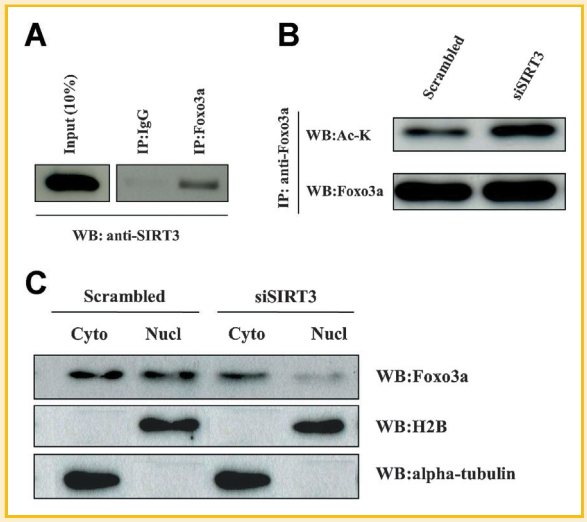

Fig. 7. SIRT3 interacts with FOXO3a. A: Foxo3a binds to SIRT3 in HOK cells. Endogenous Foxo3a in HOK cells was immunoprecipitated using a Foxo3a antibody and analyzed by Western blotting using an antibody against SIRT3. B: Knocking down SIRT3 increases endogenous Foxo3a acetylation. Acetylation levels of endogenous Foxo3a in HOK cells with or without siSIRT3 and then treated with H_2O_2 to induce protein acetylation. Foxo3a was immunopurified and probed by acetyl lysine antibody (Ac-K). C: Cytoplasmic and nuclear fractions of HOK cells with or without siSIRT3 were prepared and analyzed by Western blotting using antibodies against Foxo3a, H2B, and alpha-tubulin. Data were represented as the mean ± SD from at least three independent experiments.

the inhibition of cell growth in early passages. ANE-induced cytoinhibition appeared to be related to G_1/S phase arrest. It has previously been shown that ANE induces G_1 phase arrest in normal human oral keratinocytes [Lu et al., 2006]. In these cells, the G_1 phase of the cell cycle is responsible for growth factor-dependent signals [Blagosklonny and Pardee, 2002]. Dysregulation of the G_1 phase is advantageous to tumorigenic cell growth; as such, G_1 regulation is frequently disrupted in cancer through regulator impairment [Kang et al., 2004]. A previous study showed that ROS contributes to cell cycle progression in addition to influencing cell proliferation and transformation [Havens et al., 2006]. High levels of ROS result in DNA damage as well as damage to proteins and lipids and thereby lead to cell death, senescence or aging [Martindale and Holbrook, 2002]. In this study, there was rapid generation of ROS following ANE treatment. Early occurrence and sustained ROS generation over 24 h was likely a major reason for ANE-induced cytoinhibition. It has been postulated that ROS elicited by ANE could be an important mechanism by which these cellular responses are triggered [Lu et al., 2006], but this has not yet been elucidated.

Recent findings have supported SIRT3 involvement in cellular defenses against oxidative stress and its role in regulating cellular ROS levels [Kim et al., 2010; Qiu et al., 2010; Someya et al., 2010; Tao et al., 2010]. Constitutive expression of SIRT3 was shown to reduce ROS levels in adipocytes [Shi et al., 2005]. In addition, increased expression of SIRT3 protected myocytes from genotoxic and oxidative stress and blocked cardiac hypertrophy by activating antioxidant enzymes, such as SOD2 and catalase [Sundaresan et al., 2008, 2009]. $SIRT3^{-/-}$ mouse embryonic fibroblasts were recently shown to exhibit increased superoxide levels [Kim et al., 2010], and in vivo overexpression of *Sirt3* suppressed cellular ROS levels [Bell et al., 2011]. These studies support the notion that SIRT3 could potentially be used to combat ANE-related oxidative stress. We confirmed a significant increase in ROS levels in ANE-treated HOK cells and found that ANE was able to modulate SIRT3 expression and activation. Following ANE treatment, SIRT3 was up-regulated and activated in HOK cells. Interestingly, ROS scavengers could abrogate SIRT3 activation in that after treatment with NAC to remove ROS, the ANE-induced increase in SIRT3 activity was eliminated. Although this study implicates SIRT3-dependent regulation of ROS by ANE in HOK cells, the reciprocal interaction between ROS and SIRT3 following ANE stimulation might require further dissection. However, to our knowledge, the possible role of SIRT3 in regulating cellular ROS levels underlying ANE-treated HOK cells has not been previously investigated. To understand the role of SIRT3, we utilized siRNA oligonucleotides against SIRT3 to suppress SIRT3 expression. We found an increase in ROS as a consequence of SIRT3 knockdown. In addition, knockdown of SIRT3 increased ROS levels by reducing the expression of antioxidative genes *SOD2* and *CAT*. SIRT3 knockdown cells decreased the specific activity of SOD2 by reducing the deacetylation of SOD2, even after ANE stimulation. It is possible that increased ROS levels through ANE-stimulated SIRT3 transcription lead to SOD2 deacetylation and activation, thus supporting the role of SIRT3 in activating SOD2 to decrease cellular ROS in HOK cells. This is consistent with previous reports in which detoxification of ROS by SOD2 was demonstrated to be a direct target of SIRT3 [Qiu et al., 2010; Someya et al., 2010; Tao et al., 2010]. Interestingly, four of the eleven proteins that make up complex III in mitochondria were shown to be acetylated [Schwer et al., 2009]. It will be interesting to determine if acetylation of these subunits alters ROS generation from complex III and if any of these subunits are direct targets of SIRT3 in HOK cells. Because SIRT3 can promote SOD2 activity, we examined changes in deacetylation and enzyme activity of SOD2 after ANE stimulation in HOK cells. In association with decreased SOD2 acetylation, SOD2 enzyme activity was increased 1.9- and 2.4-fold after 6 and 24 h of ANE treatment, respectively. However, acetylation of SOD2 was slightly increased after 2 h of ANE treatment (Fig. 6A). SOD2 is a homotetrameric enzyme and forms a unique intersubunit 4-helix bundle interface important for assembly and stabilizing binding to the metal cofactor Mn^{3+} and superoxide. SOD2 activity is tightly regulated by acetylation on its lysine residues. There are 11 positively charged residues that form a ring surrounding the active site channel [Borgstahl et al., 1992; Qiu et al., 2010; Tao et al., 2010]. Lysine acetylation has recently emerged as an important, and perhaps the primary, post-translational modification to regulate mitochondrial proteins [Schwer et al., 2006; Choudhary et al., 2009]. A recent study reported that SOD2 acetylation could be increased and enzyme activity decreased by activated aryl hydrocarbon receptor (AhR) in tetracycline-inducible CA-AhR TG mice [He et al., 2013]. AhR is a ligand activated xenobiotic receptor expressed in most tissues [Carver et al., 1994; Gu et al., 2000]. AhR can be activated by numerous environmental contaminants and endogenous ligands [Nebert et al., 2004; Beischlag et al., 2008; Nguyen and

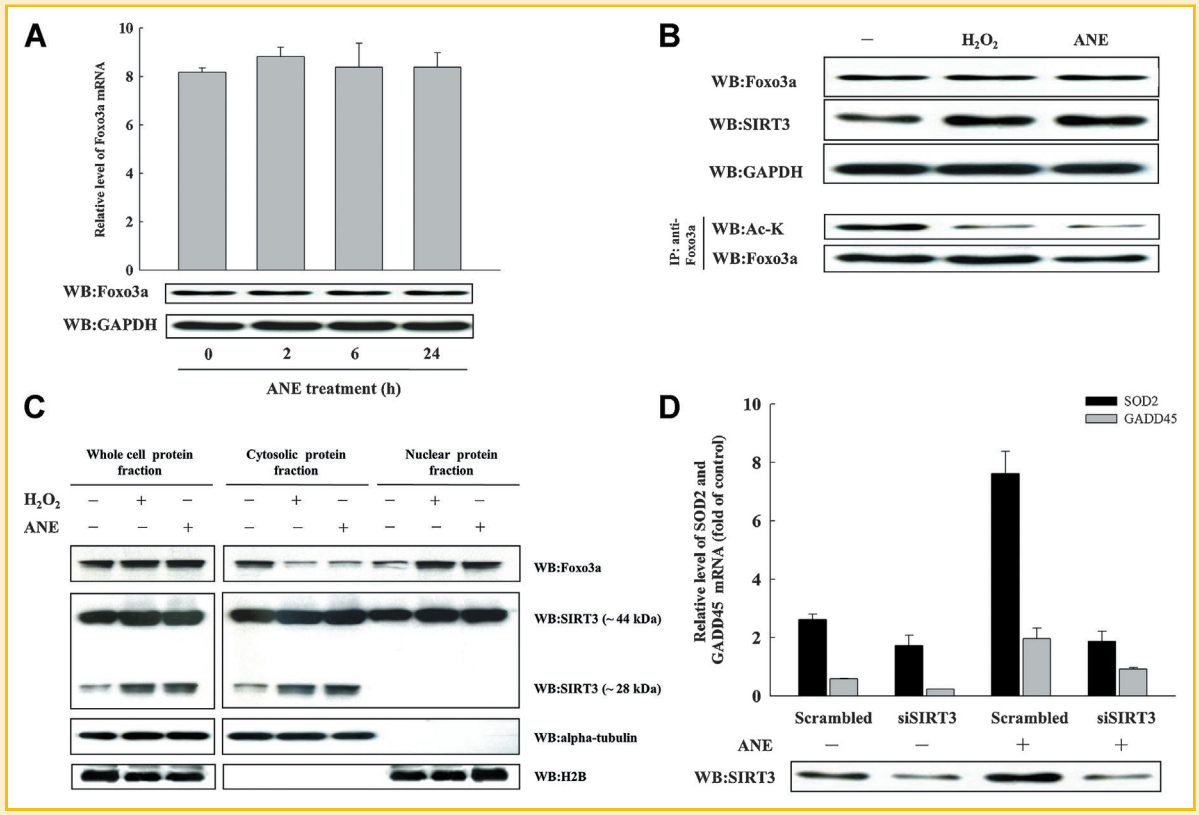

Fig. 8. ANE-induced ROS stimulates SIRT3 expression to deacetylate and activate Foxo3a. A: Foxo3a expression after ANE treatment. Expression patterns of mRNA and protein levels of Foxo3a in HOK cells after ANE stimulation at various time points were determined by real-time PCR and Western blotting, respectively. B: ROS stress induces deacetylation of Foxo3a. SIRT3 protein levels and Foxo3a expression in HOK cells following ANE or H_2O_2 treatment for 24 h were determined. Acetylation levels of endogenous Foxo3a in HOK cells and then treated with ANE or H_2O_2 to induce protein acetylation. Foxo3a was immunopurified and probed by an acetyl lysine antibody (Ac-K). C: Nuclear localization of Foxo3a after ANE stimulation. Cell lysates and cytoplasmic and nuclear fractions of HOK cells following ANE or H_2O_2 treatment for 24 h were prepared and analyzed by Western blotting. D: ANE activates Foxo3a through SIRT3. mRNA levels of Foxo3a targeted genes SOD2 and GADD45 in HOK cells with or without siSIRT3. Data are represented as the mean ± SD from at least three independent experiments.

Bradfield, 2008; Opitz et al., 2011], inferring that ANE could be associated with AhR activation. We assume that ANE stimulation may lead to AhR activation and that activation of AhR increases SOD2 acetylation in a very short time period. ANE-induced ROS then stimulates SIRT3 expression, leading to increased deacetylation and activation of SOD2. However, it is still unclear whether ANEs are associated with AhR activation and will require further study.

A recent study showed that SOD2 and catalase expression were controlled by the Foxo group of transcription factors [Kops et al., 2002; Nemoto and Finkel, 2002]. Another report showed that SIRT3 could activate Foxo3a by promoting its nuclear localization [Sundaresan et al., 2009]. In this study, we observed that Foxo3a and SIRT3 were able to bind to each other *in vivo*. After treatment with H_2O_2 to induce acetylation of Foxo3a, SIRT3 deacetylated Foxo3a in an NAD-dependent manner, but Foxo3a deacetylation was reduced following SIRT3 silencing. We investigated whether deacetylation of Foxo3a leads to its translocation from the cytoplasm to the nucleus and found that nuclear translocation of Foxo3a was eliminated after SIRT3 knockdown. These data suggest that SIRT3 is necessary for deaceylation of Foxo3a. Therefore, alterations in Foxo3a function by inhibition of Foxo3a activity or its transcriptional activity through SIRT3 knockdown might result in increased oxidative stress and an imbalance between proliferation and apoptosis. Finally, we investigated whether deacetylation of Foxo3a activates its target genes, *SOD2* and *GADD45*, which are involved in DNA replication and repair [Kops et al., 2002; Tran et al., 2002b; Li et al., 2006]. SOD2 and GADD45 expression were significantly induced by ANE stimulation. Additionally, SIRT3 knockdown decreased both the expression of SOD2 and GADD45, even after ANE stimulation. It is possible that increased Foxo3a transcriptional activity through ANE-stimulated SIRT3 transcription lead to Foxo3a deacetylation and activation, thus providing evidence that SIRT3 is necessary for deacetylation of Foxo3a to activate its target genes.

Posttranslational modifications of proteins are important in regulating their functions in health and disease. Recent studies have suggested a role for SIRT3 as a tumor suppressor. Genetic deletion of SIRT3 was shown to push mouse embryonic fibroblasts in the direction of oncogenic transformation, and SIRT3$^{-/-}$ mouse embryonic fibroblasts exhibited stress-induced genomic instability [Kim et al., 2010]. While activation of both oncogenes *Myc* and *Ras* is

needed to transform an immortalized fibroblast into a tumor-forming cell, the genetic deletion of SIRT3 reduced that requirement to only activation of either *Myc* or *Ras*. Thus, SIRT3 functions as a tumor suppressor [Schumacker, 2010]. In addition, overexpression of *SIRT3* was shown to decrease tumorigenesis in xenografts, even when induction of SIRT3 occurred after tumor initiation [Bell et al., 2011]. These data suggest that the chronic increase in mitochondrial ROS stress might result in mitochondrial or genomic DNA damage and altered intracellular metabolism. In addition, SIRT3 was decreased in commercially obtained tissue microarray samples of human breast cancer, glioblastoma, prostate, head and neck and other cancers based on a review of gene expression data from other sources [Kim et al., 2010]. Similarly, we found that ROS levels were increased in SIRT3 knockdown HOK cells by decreasing expression of antioxidant enzymes, such as SOD2 and CAT. Loss of SIRT3 activity suppresses Foxo3a, which is the transcription factor of an antioxidant enzyme, and SIRT3-mediated deacetylation of Foxo3a promotes its nuclear localization. Thus, the loss of SIRT3 activity leads to an increase in cellular ROS signaling, thereby enhancing tumorigenesis.

In summary, our findings reveal that SIRT3 is necessary for mediating the activation of ROS-detoxifying enzymes by promoting Foxo3a translocation to the nucleus after ANE stimulation. This suggests that SIRT3 may be a key regulator of cellular defense mechanisms during oxidative stress. Future studies will examine the therapeutic potential of manipulating SIRT3 expression or activity in oral keratinocytes to ameliorate the manifestations of oxidative stress.

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