SIRT6 Promotes COX-2 Expression and Acts as an Oncogene in Skin Cancer

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Abstract
SIRT6 is a SIR2 family member that regulates multiple molecular pathways involved in metabolism, genomic stability, and aging. It has been proposed previously that SIRT6 is a tumor suppressor in cancer. Here, we challenge this concept by presenting evidence that skin-specific deletion of SIRT6 in the mouse inhibits skin tumorigenesis. SIRT6 promoted expression of COX-2 by repressing AMPK signaling, thereby increasing cell proliferation and survival in the skin epidermis. SIRT6 expression in skin keratinocytes was increased by exposure to UVB light through activation of the AKT pathway. Clinically, we found that SIRT6 was upregulated in human skin squamous cell carcinoma. Taken together, our results provide evidence that SIRT6 functions as an oncogene in the epidermis and suggest greater complexity to its role in epithelial carcinogenesis. Cancer Res; 74(20); 5925–33. ©2014 AACR.

Introduction
Skin cancer is the most common cancer in the United States (1, 2). The pathogenesis of skin cancer requires both genetic and nongenetic molecular alterations. In particular, important oncogenic or tumor-suppressive alterations may increase cell survival and proliferation, impair DNA repair and checkpoint activation, and induce inflammation in vivo (3–5). Targeting these molecular pathways has been demonstrated to hold promise for skin cancer prevention (3, 6).

SIRT6 is an antiaging protein important for many aspects of organismal health (7–9). SIRT6 knockout mice have displayed genomic instability and several phenotypes of accelerated premature aging (10). Overexpressing SIRT6 extends lifespan in male mice but not in female mice (11). Ablation of neural SIRT6 causes obesity in mice (12). SIRT6 promotes reprograming of induced pluripotent stem cells from humans (13), and also regulates skin aging by modulating collagen metabolism in dermal fibroblasts (14).

At the molecular and cellular level, SIRT6 regulates multiple molecular pathways to modulate gene transcription, glucose homeostasis, DNA repair, and telomere integrity (7–9). SIRT6 is a protein deacetylase that deacetylates Histone H3 lysine 9 (H3K9), required for telomere maintenance (9, 15). It suppresses NF-kB by interacting with the NF-kB RELA subunit and deacetylating H3K9 to delay the signs of aging (16), and it blocks IGF-AKT signaling to protect against development of cardiac hypertrophy (5). It regulates glucose homeostasis (17, 18), repressing hypoxia-inducible factor-1a (19) and activating acetyltransferase GCN5 (20). In addition, SIRT6 regulates double-strand break (DSB) repair through recruiting SNF2H to DNA strand breaks, (21), deacetylating the DSB resection protein CtIP (22), activating PARP1 under oxidative stress (23), and stabilizing DNA-dependent protein kinase (24). SIRT6 reverses the decline of homologous recombination repair during replicative senescence (25).

As a positive regulator of genomic integrity, SIRT6 is predicted to act as a tumor suppressor. Indeed, it is found to suppress tumorigenesis inintestinal and liver cancer in mice (26–28). However, SIRT6 has been implicated as an oncogene in skin cancer (29) and prostate cancer (30). It seems that its function may be tissue- and context dependent. A mouse model with tissue-specific SIRT6 deletion is needed to define the function of SIRT6 in carcinogenesis.

Here, we report that skin-specific SIRT6 deletion inhibits chemical skin tumorigenesis. SIRT6 promotes the expression of the proinflammatory and prosurvival protein COX-2 through suppressing AMPK signaling, and increases cell survival and proliferation. UVB induces SIRT6 expression through activating the AKT pathway. SIRT6 is upregulated in human squamous cell carcinoma (SCC). Our findings demonstrate that SIRT6 is an oncogene in keratinocytes and provide new molecular insights into its pivotal function in epidermal carcinogenesis.

Materials and Methods
Human samples
All human specimens were studied after approval by the University of Chicago Institutional Review Board (Chicago, IL).
The SIRT6 protein and mRNA levels were analyzed in sun-protected normal skin and sun-exposed SCC samples (n = 6) by immunoblotting and RT-PCR analysis, respectively. All SCC samples were examined by a dermatopathologist to determine the SCC diagnosis.

**SIRT6 WT and cKO mice and DMBA/TPA treatments**

All animal procedures have been approved by the University of Chicago Institutional Animal Care and Use Committee. The mice with floxed Sirt6 (FVB background; ref. 18) were bred to mice expressing Cre recombinase driven by the K14 promoter (Jackson Laboratory, in B6 background) to generate skin keratinocyte-specific SIRT6 wild-type (WT) and skin-specific homozygous SIRT6 knockout (cKO) mice. Mice (n = 15 for each group, female, in a background with 50% B6 and 50% FVB) were shaved one day before the initial 7,12-dimethylbenz[a]anthracene (DMBA) treatment and later as needed. Mice were treated with a single dose of 50 μg DMBA in 100 μL acetone. One week after the DMBA application, 5 μg of 12-O-tetradecanoylphorbol-13-acetate (TPA; in 200 μL acetone) was applied topically twice a week for up to 30 weeks. The number of tumors per mouse was recorded weekly.

**Cell culture, UVB treatment, siRNA/plasmid transfection, and adenoviral infection**

Normal human epidermal keratinocytes (NHEK) were obtained from Clonetics (Lonza) and cultured in KGM Gold BulletKit medium (Clonetics, Lonza) according to the manufacturer’s instructions. NHEK cells were cultured for less than four passages. The cells at a confluence of 60% to 80% were treated with DMBA (0, 10, 100 μmol/L) or TPA (0, 100, or 500 nmol/L) for 24 hours, actinomycin D for 0.5, 1, 2, 6, and 24 h, or UVB at 72 hours after transfection with siRNA or combination of siRNA and plasmid expressing COX-2. For the UVB treatment, a Stratalink 2400 equipped with 321-mm UVB bulbs (Stratagene; UVC 0%, UVB 51%, and UVA 49%) was used. The UV exposure was performed in PBS after washing the cells with PBS twice to avoid the photosensitization effect of components in the culture medium on the cells. Cells were transfected with negative control siRNA or vector, siRNA-targeting SIRT6 or COX-2 (SMARTpool: ON-TARGETPlus SIRT6 or PTGS2 siRNA, Dharmacon), or a plasmid-expressing COX2 (Origene) using Amxa Nucleofector according to the manufacturer’s instructions as described in our recent studies (31). Cells at a confluence of 40% to 50% were infected with an adenoviral vector-expressing wild-type SIRT6 (ad-SIRT6) or empty vector as described previously (32). No cell line was used.

**Hematoxylin and eosin staining and Ki67 IHC**

Hematoxylin and eosin (H&E) staining and Ki67 IHC were performed by the Immunohistochemistry core facility at the University of Chicago. Briefly, specimens of skin tissue were fixed in 10% formalin and then embedded with paraffin. The paraffin sections were stained with H&E for histologic observations. Image acquisition of H&E-stained tissue sections was performed using a CRI Panoramic Scan Whole Slide Scanner. Scanned slides were viewed and analyzed with Panoramic Viewer (3DHISTECH), where the thickness of the epidermis was determined by hand-drawn line segments connecting the intact stratum corneum to the dermal–epidermal junction. Twenty line segments were drawn on each of the five sections per slide for 100 thickness measurements per tissue. The mean and SE were used for determining the response of mouse epidermis to DMBA/TPA or UVB. The number of Ki67^+ cells was quantified by determining the total number of Ki67^+ cells per ×40 magnification for five areas randomly.

**Western blotting**

All proteins were extracted with RIPA lysis buffer and protein concentrations were determined using the BCA assay (Pierce). For mouse skin, the tissue was homogenized in liquid nitrogen and then lysed in RIPA lysis buffer. Equal amounts of protein were subjected to electrophoresis. Western blotting was performed as described previously (32, 33). Antibodies used included COX-2, AKT, p53, p21, Chk1, Chk2, XPC, DDB1, DDB2, H2AX, β-actin, GAPDH (Santa Cruz Biotechnology), SIRT6, p-AKT (serine 473), cleaved caspase-3, p-AMPK, p-Chk1, p-Chk2 (Cell Signaling Technology), acetylated k382 p53 (ac-p53; Abcam), and ac-H3K9 (Sigma).

**Sub-G1 flow-cytometric analysis**

Sub-G1 flow-cytometric analysis was performed to determine apoptosis as described previously (34). Briefly, cells were fixed and stained with propidium iodide, and the percentage of cells at the sub-G1 phase was quantified by flow cytometry.

**RNA extraction and real-time PCR**

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instruction as in our recent studies (35). qRT-PCR assays were performed using ABI7300 (Applied Biosystems). Real-time RT-PCR fluorescence detection was performed in 96-well plates with SYBR Green PCR Master Mix (Applied Biosystems). Amplification primers for SIRT6 and COX-2 were purchased from Origene, 5'-ACTGGAACGGTGAAGGTGACA-3' (forward) and 5'-ACTG-0-ATGGCAAGG- (reverse) for β-actin. The threshold cycle number (Ct) for each sample was determined in triplicate. The Ct values for SIRT6 and COX-2 were normalized against β-actin as described previously (35).

** Luciferase reporter assays**

The plasmid mixtures contained 1 μg of COX-2 promoter luciferase construct (COX-2-luc in pGL3 vector, kindly provided by Dr. De Ferrari from Universidad Andres Bello, Santiago-Chile) and 0.025 μg of pRL-TK (Promega, used as a transfection efficiency control). They were transfected with electroporation according to the manufacturer’s protocol. The luciferase activity was measured as described previously (35).

**Statistical analyses**

Statistical analyses were performed using Prism 5 (GraphPad software). Kaplan–Meier survival estimates and log-rank tests were used to evaluate tumor onset in mice. Data were expressed as the mean of three independent experiments and
analyzed by Student t test. A P value of less than 0.05 was considered statistically significant.

Results

SIRT6 is upregulated in human skin SCC and is an oncogene in mouse skin tumorigenesis

To determine whether SIRT6 has a role in skin cancer, we assessed the difference in SIRT6 protein and mRNA abundance in sun-protected normal human skin and human SCC derived from sun-exposed areas. Compared with normal human skin, SIRT6 protein and mRNA levels were upregulated in SCC (Fig. 1A and B), suggesting that SIRT6 is an oncogene in human skin SCC. To determine its role in skin tumorigenesis, SIRT6 WT and skin-specific conditional knockout mice (cKO) were treated with the chemical carcinogens DMBA and TPA to allow tumor development (Fig. 1C). SIRT6 WT mice treated with DMBA/TPA developed tumors at 7 weeks after TPA (Fig. 1D). As compared with WT mice, mice with epidermal SIRT6 ablation showed reduced tumorigenesis (Fig. 1D) and tumor multiplicity (Fig. 1E), indicating that loss of SIRT6 suppresses skin tumorigenesis.

SIRT6 loss suppresses proliferation and epidermal hyperplasia in mouse skin

Deregulated cell proliferation is critical for tumor formation. To determine whether SIRT6 plays a role in DMBA/TPA-induced cell proliferation in vivo, we assessed the difference in epidermal thickness and the number of Ki67+ epidermal cells in normal skin, nontumor skin, and tumors from SIRT6 WT and cKO mice. Treatment of WT mice with DMBA-TPA increased epidermal thickness (Fig. 2A) and Ki67+ cells (Fig. 2B) in nontumor skin and tumors compared with untreated skin. However, SIRT6 ablation reduced DMBA-TPA–induced epidermal hyperplasia and Ki67+ cells in SIRT6 cKO groups (Fig. 2A and B). In addition, we treated WT and SIRT6 cKO mice with UVB radiation to further confirm the effect of SIRT6 on cell proliferation in vivo. UVB irradiation increased epidermal hyperplasia and Ki67+ cells, whereas SIRT6 ablation decreased UVB-induced epidermal hyperplasia (Fig. 2C and D) and the number of Ki67+ epidermal cells in UVB-irradiated mice (Fig. 2E and F). These data indicate that SIRT6 loss suppresses DMBA-TPA and UVB-induced cell proliferation in vivo. However, SIRT6 did not affect UVB-induced DNA damage repair or checkpoint signaling (Supplementary Fig. S1A–S1E), consistent with previous findings (10).

SIRT6 regulates COX-2 expression

COX-2 functions as an oncogene and promotes cell proliferation and survival (36, 37). To determine whether SIRT6 has a role in the function of SIRT6, we first analyzed the effect of SIRT6 inhibition on COX-2 expression. As compared with negative control (NC), COX-2 mRNA and protein levels were decreased in NHEK cells transfected with siRNA-targeting SIRT6 (siSIRT6; Fig. 3A and B). COX-2 protein levels were reduced in DMBA/TPA-treated skin of SIRT6 cKO mice as compared with WT mice (Fig. 3C). Overexpression of SIRT6 increased COX-2 protein abundance (Fig. 3D).
skin and tumor tissues after treatment with DMBA and TPA, COX-2 protein levels were reduced in cKO mouse skin as compared with its WT counterparts (Fig. 3E). Furthermore, inhibition of SIRT6 decreased COX-2 expression induced by UVB (Fig. 3F), DMBA (Fig. 3G), or TPA (Fig. 3H).

**SIRT6 promotes cell survival by regulating COX-2 expression**

Apoptosis plays an important role in both carcinogenesis and cancer treatment. To determine whether SIRT6 plays a role in apoptosis in vitro, we used a flow cytometry assay to measure the sub-G1 percentage (%) of apoptotic cells in NHEK cells transfected with negative control or siRNA-targeting SIRT6 following sham or UVB radiation. SIRT6 inhibition significantly increased apoptosis (Fig. 4A and B). The active form of caspase-3 (cleaved caspase-3), another marker for apoptosis, was increased in siSIRT6-transfected NHEK cells as compared with cells transfected with negative control siRNA (Fig. 4C). These findings suggested that inhibition of SIRT6 sensitized cells to UVB-induced apoptosis. Knockdown of COX-2 decreased UVB-induced AKT phosphorylation (Fig. 4D) and increased UVB-induced caspase-3 cleavage (Fig. 4E). Restoration of COX-2 expression in siSIRT6-inhibited NHEK cells decreased UVB-induced caspase-3 cleavage upon SIRT6 inhibition (Fig. 4F and G). These findings suggested that SIRT6 knockdown suppresses cell survival through downregulating COX-2.

**SIRT6 regulates COX-2 mRNA stability by inhibiting the AMPK pathway**

To determine how SIRT6 regulates COX-2 expression, we used the luciferase reporter assay to determine whether SIRT6 regulates COX-2 at the transcriptional level. We found that SIRT6 knockdown had no effect on COX-2 promoter activity in NHEK cells (Fig. 5A). To determine whether SIRT6 regulates COX-2 mRNA stability, we treated NC- or siSIRT6-transfected NHEK cells with the RNA synthesis inhibitor actinomycin D (1 μmol/L) over a time course. As compared with NC-transfected cells, siSIRT6-transfected cells showed reduced COX-2 mRNA stability (Fig. 5B). It has been shown that UVB increases COX-2 mRNA stability through suppressing the AMPK pathway (38), so our next question was whether AMPK plays a role in SIRT6 regulation of COX-2 mRNA stability. When we inhibited SIRT6, phosphorylation of AMPK and its substrate ACC increased (Fig. 5C). The AMPK inhibitor Compound C (CC, 10 μmol/L) increased COX-2 protein abundance in both NC- and siSIRT6-transfected cells (Fig. 5D). These findings indicate that SIRT6 regulates COX-2 mRNA stability by inhibiting the AMPK pathway.

**UVB upregulates SIRT6 through activating AKT**

To further determine the role of SIRT6 in skin cancer, we examined the effect of UVB radiation on SIRT6. We found that at 24-hour after UVB, the protein levels of SIRT6 and COX-2...
increased (Fig. 6A). LY294002, a PI3K/AKT inhibitor, inhibited UVB-induced upregulation of SIRT6 at the protein and mRNA levels (Fig. 6B and C). Knockdown of AKT1 inhibited AKT phosphorylation and UVB-induced SIRT6 upregulation (Fig. 6D). Inhibition of SIRT6 also suppressed AKT phosphorylation (Fig. 6E). UVB-induced SIRT6 expression and AKT phosphorylation likely form a positive feedback system in which SIRT6 regulates COX-2 mRNA stability by inhibiting AMPK activation. This regulation of COX-2 then affects inflammation and survival, which may contribute to skin carcinogenesis (Fig. 6F).

Discussion

The mammalian sirtuin family consists of seven members, SIRT1-7. Sirtuins have been investigated in cancer pathogenesis (39). SIRT1 is both tumor suppressive and oncogenic depending on the context and gene dose (39–42). In contrast, SIRT2 is reported to be a tumor suppressor in mice and downregulated in human skin cancer (43, 44). In this study, we analyzed the role of SIRT6 in skin tumorigenesis. SIRT6 is upregulated in human SCC, and skin-specific SIRT6 ablation suppresses skin tumorigenesis. Furthermore, SIRT6 loss inhibits cell survival and proliferation in vivo and downregulates COX-2 at the posttranscriptional level. UVB radiation increases SIRT6 expression through activating AKT. Our findings demonstrate the oncogenic role of SIRT6 in skin cancer (Fig. 6F) and suggest SIRT6 as a potential target for developing chemopreventive strategies to reduce the skin cancer burden.

The function of SIRT6 in cancer is likely context- and tissue specific. SIRT6 is shown to be a tumor suppressor in liver...
cancer and intestinal cancer; it acts as a tumor suppressor by controlling cancer metabolism (26). SIRT6 is downregulated in human pancreatic and colon cancers. It functions as a regulator of aerobic glycolysis by corepressing MYC transcriptional activity (26). SIRT6 suppresses liver cancer initiation through suppressing the oncogene survivin (27). It is downregulated in human liver cancer and controls oncogenic molecular pathways (28). Overexpression of SIRT6 induces apoptosis in cancer cells but not in normal cells (45). This tumor-suppressive function is also supported by the positive role of SIRT6 in DNA repair and telomere integrity (7–9).

Here, we show that SIRT6 acts as an oncogene in the skin, promoting survival and proliferation. Indeed, SIRT6 was shown to be upregulated in human SCC and is a target of miR-34a in keratinocytes (29). SIRT6 is upregulated in human prostate cancer (30). In keratinocytes, we show that SIRT6 promotes the expression of COX-2, an enzyme involved in inflammation, proliferation, and survival. The functions of SIRT6 in cancer may depend on the tissue-specific molecular profiles and carcinogens.

COX-2 is an inducible enzyme catalyzing the first step in the synthesis of prostaglandins and acts as a proinflammatory factor. It has been identified as an oncogene in skin cancer and is upregulated in human skin cancer (46). In both chemical and UV carcinogenesis models, genetic ablation of COX-2 suppresses skin tumorigenesis (47, 48). COX-2 promotes cell proliferation and survival following UV radiation (36, 37). Recent studies have shown that intrinsic keratinocyte COX-2 activity plays a major role in skin cancer (49). Our findings elucidate the critical role of SIRT6 in the regulation of the oncogenic COX-2 pathway.

COX-2 is regulated at both the transcriptional and posttranscriptional level. For example, COX-2 expression is positively regulated by the mRNA stability factor HuR (50). HuR is mainly localized in the nucleus in unstimulated cells. The nuclear localization of HuR is regulated by AMPK-induced activation of Importin a1 (51). AMPK activation reduces cytoplasmic HuR and HuR association with the target transcripts (52). UVB radiation inhibits the AMPK pathway, thus promoting COX-2 mRNA stability (38). We found that SIRT6 knockdown increased AMPK activation. It is possible that SIRT6 loss reduces ATP levels due to upregulated glycolysis and diminished mitochondrial respiration (19), despite increased glucose uptake (17, 18), and therefore stimulates an energy stress response that can lead to AMPK activation.
Another possible mechanism is SIRT6-mediated protein lysine fatty acylation (53). SIRT6 promotes the secretion of TNFα by removing the fatty acyl modification at K19 and K20 of TNFα (53). Previous studies have shown that TNFα induces COX-2 transcription through the NF-kB pathway (54); this pathway is unlikely to be involved in SIRT6-regulated COX-2 abundance, because SIRT6 regulates the mRNA stability of COX-2 (Fig. 5B). Recent work has demonstrated that TNFα suppresses AMPK signaling through transcriptional upregulation of protein phosphatase 2C (55), which may have a role in the regulation of COX-2 mRNA stability by SIRT6.

SIRT6 acts as a downstream target of AKT activation following UVB radiation. SIRT6 is increased in human SCC and by UVB radiation (Figs. 1A and 6). Previous studies have shown that nutrient starvation increases SIRT6 protein levels (56). A recent report has shown that miR-34a targets and suppresses SIRT6 expression in keratinocyte differentiation (29). We found that AKT signaling is required for UVB-induced SIRT6 upregulation. It is possible that AKT mediates UVB-induced SIRT6 expression through c-fos because (i) AKT activation induces c-fos expression and activation (57), and (ii) c-fos induces SIRT6 transcription (27). Given that UVB-induced COX-2 upregulation crosstalks with the AKT signaling (37, 58), it is possible that SIRT6 and AKT/COX-2 signaling forms a signaling network in skin carcinogenesis. Previous studies have shown that SIRT6 protects against cardiac hypertrophy by suppressing the IGF-AKT pathway in the heart (5). It is possible that the crosstalk between SIRT6 and AKT is cell-type specific and requires further investigation. Our findings...
here underscore a critical role of SIRT6 in UVB damage response and skin tumorigenesis. In summary, we have shown that SIRT6 upregulates COX-2 levels and acts as an oncogene in skin carcinogenesis. SIRT6 ablation in skin suppresses skin tumorigenesis and epidermal hyperplasia without affecting UVB-induced DNA damage repair and checkpoint signaling. SIRT6 is induced by UVB radiation. Our findings may add new molecular insights into the mechan-ism of skin carcinogenesis and provide useful tools for the development of mechanism-based cancer prevention strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: W. Han, N.R. Sundaresan, M.P. Gupta, Y.-Y. He Development of methodology: M. Ming, W. Han, B. Zhao, N.R. Sundaresan, M.P. Gupta, Y.-Y. He Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.P. Ming, W. Han, B. Zhao, N.R. Sundaresan Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Ming, W. Han, Y.-Y. He

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Keratinocyte SIRT6 as an Oncogene


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