SIRT3 inhibits prostate cancer by destabilizing oncoprotein c-MYC through regulation of the PI3K/Akt pathway

Yizhou Quan¹, Naitao Wang¹, Qianqian Chen¹, Jin Xu¹, Wei Cheng², Meijuan Di³, Weiliang Xia¹ and Wei-Qiang Gao¹,⁴

¹ State Key Laboratory of Oncogenes and Related Genes, Renji-MedX Clinical Stem Cell Research Center, Ren Ji Hospital, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai, China
² Department of Urology, First People’s Hospital of Xiaoshan, Hangzhou, Zhejiang, China
³ Department of Pathology, First People’s Hospital of Xiaoshan, Hangzhou, Zhejiang, China
⁴ Collaborative Innovation Center of Systems Biomedicine, Shanghai, China

Correspondence to: Weiliang Xia, email: wlxia@sjtu.edu.cn
Wei-Qiang Gao, email: weiqgao@yahoo.com

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ABSTRACT

SIRT3 is involved in aging-related diseases including cancer, but its role in prostate cancer and detailed regulatory function are not known. We found that SIRT3 was moderately down-regulated in prostate carcinomas. Overexpression of SIRT3 by lentiviral transfection inhibited prostate cancer growth both in vitro and in vivo, whereas knockdown of SIRT3 increased prostate tumor growth. Mechanistically, the tumor suppression effect of SIRT3 was achieved via its inhibition of the PI3K/Akt pathway. Notably, upregulation of SIRT3 suppressed the phosphorylation of Akt, leading to the ubiquitination and degradation of oncoprotein c-MYC; this could be attenuated by constitutive activation of PI3K/Akt signaling. Collectively, our results unveiled SIRT3’s tumor suppressive function and the underlying mechanism in prostate cancer, which might provide therapeutic implications for the disease.

INTRODUCTION

Prostate cancer is the most common, and second most deadly cancer type for US men [1]. Its occurrence in developing countries including China is also rapidly rising [2]. Patients are likely to relapse after primary therapy [3], and have limited treatment options. Uncontrolled progression of prostate cancer poses a critical challenge in the clinic [4, 5], and clearly it is needed to elucidate the mechanisms of prostate cancer progression and find new therapeutic targets.

Oncogenic genes such as c-MYC that are expressed at high levels in many types of cancers are tumor-inducing factors that prevent programmed cell death and cause uncontrolled cell proliferation. Strategies towards the inhibition of oncogenic genes expressions are actively pursued [6-8]. However, oncoproteins like c-MYC are not readily druggable. Hence, finding new ways and understanding the underlying mechanism of inhibiting oncogenic genes is urgently needed.

Mainly located in the mitochondrion, SIRT3 belongs to the Sirtuin family and is a longevity protein that could extend lifespan by suppressing oxidative stress [9]. Thus far antioxidant therapies for cancer patients have not been successful because antioxidants could hardly access to the mitochondria-localized pools of ROS [10, 11]. In this regard, therapies that directly suppress mitochondria-derived ROS could be an ideal approach [10]. Initially identified as a tumor suppressor in breast cancer, SIRT3 maintained the integrity of mitochondria during stress and Hif1α destabilization [12, 13]. However, little is known about SIRT3’s function in prostate cancer. In this study, we report that SIRT3 could act as a prostate tumor suppressor through inhibition of the PI3K/Akt pathway, resulting in ubiquitination and degradation of oncoprotein c-MYC. Up-regulation of SIRT3 might serve as new therapeutic strategies for prostate cancer.
RESULTS

SIRT3 is moderately down-regulated in human prostate carcinoma and positively correlates with patient survival

To explore the relationship between SIRT3 expression levels and prostate cancer progression, we first examined multiple microarray datasets in the Oncomine Database (www.oncomine.com). Analysis of a dataset containing 19 human clinical specimens showed that SIRT3 mRNA level in prostate carcinoma (PCa) was much lower than that in normal prostate tissues (Figure 1A). A second dataset including 96 human samples also revealed down-regulation of SIRT3 mRNA in PCa relative to normal tissue or prostatic intraepithelial neoplasia (PIN) (Figure 1B). To provide additional evidence in support of this notion, we performed immunohistochemical (IHC) staining of SIRT3 in primary tumors of prostate cancer patients (n=109, of which 32 biopsies were benign tissues, and 77 biopsies were tumor tissues). IHC microscopy analysis revealed that most of SIRT3...
信号被定位在非癌组织的上皮细胞中（图1C）。样品被评分，根据细胞质与胞膜的百分比，SIRT3阳性细胞，以及被随机分为低(10%-20%阳性)、中(20%-50%阳性)和高(>50%阳性)组。在非癌组织样本中，SIRT3高评分组的百分比下降到22%（16/74）与SIRT3低评分组的58%（43/74）（图1D）。对临床样本的特征的详细描述和SIRT3抑制在补充表1中被提供。这些结果支持SIRT3抑制在前列腺癌样本中。

为了确定SIRT3表达是否与患者的生存有关，我们使用了来自Oncomine的基因表达数据集和Kaplan-Meier生存分析。基因拷贝数在前列腺癌样本中在分化患者的样本中显著性地长于其它组（图1E）。这些结果表明SIRT3与前列腺癌患者临床结果呈负相关。

**Overexpression of SIRT3 inhibits prostate cancer cell proliferation in vitro and in vivo**

为了探索SIRT3在前列腺癌中的功能，我们首先生成了SIRT3稳定过表达的，去势抵抗型前列腺癌（CRPC）细胞系（C42B-SIRT3和PC3-SIRT3）。免疫荧光显微镜和Western blot分析确认了SIRT3在前列腺癌样本中显著性地高于其对照细胞（图2A & 2B）。SIRT3过表达显著性地降低了前列腺癌细胞的形成和在体外细胞增殖（图2C & 2D）。细胞生长抑制由SIRT3也观察到在三维软琼脂形成和细胞存活率的前列腺癌细胞系（图3A & 3B）。SIRT3敲低在这些两种细胞系中被确认（图3C-3E）。此外，SIRT3敲低也促进了前列腺癌细胞生长，如图3F & 3G所示。这些结果证实了SIRT3抑制在培养基中的SIRT3表达在体内抑制前列腺癌细胞增殖（图3H-3J）。

**SIRT3 induces destruction of oncoprotein c-MYC in prostate cancer cells**

为了理解SIRT3机制，我们专注于过表达的细胞上的SIRT3。作为过表达的细胞，c-MYC已被报道能维持肿瘤的进展和加速肿瘤的生长在不同的癌组织类型[16-18]。此外，c-MYC的表达是被上调的在前列腺癌细胞和高度相关的肿瘤的进展[19-21]。SIRT3被SIRT3抑制前列腺癌细胞增殖（图2），推测SIRT3可能会通过抑制过表达的细胞c-MYC。首先，我们使用了来自Oncomine的数据集和发现的SIRT3 mRNA表达水平在与低c-MYC表达(图4A)相关。过表达的细胞c-MYC水平显著性地高于低c-MYC表达水平(图4B)。这些结果表明了SIRT3和c-MYC之间的逆相关。

**Knockdown of SIRT3 accelerates prostate cancer cell proliferation in vitro and in vivo**

我们进一步通过SIRT3抑制SIRT3过表达前列腺癌细胞和控制细胞。确实，SIRT3过表达c-MYC在SIRT3过表达前列腺癌细胞和控制细胞中得到了抑制(图4C)和在体(图4D & 4E)。相反，SIRT3敲低促进了c-MYC在SIRT3过表达前列腺癌细胞的降解和 ubiquitination (图4F)。这些数据表明SIRT3可以抑制c-MYC在前列腺癌。

为了进一步阐明机制，我们通过SIRT3抑制c-MYC水平，我们试图测试SIRT3是否也能影响c-MYC蛋白的稳定性。SIRT3抑制c-MYC被报道能被ubiquitin-mediated proteolysis在不同的癌组织[22-24]。我们对SIRT3的SIRT3过表达和SIRT3抑制在前列腺癌中的作用进行了探索。SIRT3过表达的c-MYC蛋白显著性地减少在SIRT3过表达前列腺癌细胞中，与细胞数比较。在c-MYC蛋白水平上，SIRT3抑制c-MYC蛋白的稳定性被提出了。这些结果表明SIRT3可能抑制c-MYC并抑制ubiquitin-mediated proteolysis。
Figure 2: Overexpression of SIRT3 suppresses prostate cancer cell growth. Immunofluorescence microscopy (A) and Western blot (B) analysis of SIRT3 expression in stably overexpressed (SIRT3) and control (Vector) cells (C42B and PC3 PCa cell lines). Bar =20 μm. (C) Clone formation assay of SIRT3 stably overexpressed (SIRT3) and control (Vector) cells. Left panel: representative photographs of cell colonies; right panel: bar graph summarizing the number of colonies per field (top: C42B; bottom: PC3) (**p < 0.01, ***p < 0.001, Student’s t-test; n=6). (D) Cell viability assay of SIRT3 stably overexpressed (SIRT3) and control (Vector) cells (top: C42B; bottom: PC3. ***p < 0.001, two-way ANOVA, followed by post-hoc tests). (E) In vivo tumor formation assay of SIRT3 overexpressed vs. control vector transfected C42B prostate cancer cells. The tumor volumes (***p < 0.001, two-way ANOVA, followed by post-hoc tests) and tumor weights (***p < 0.001, Student’s t-test) between two groups were analyzed. Dissected tumors from mice at the end of the experiment were also photographed. Data of Fig D, E (tumor volume) are expressed as means ±SD. Data of rest figures are expressed as means ±SEM.
Figure 3: Knockdown of SIRT3 promotes prostate cancer proliferation. DU145 cells stably transfected with sh-SIRT3 lentivirus (sh1 and sh2) or scrambled control virus (scramble) were subjected to various assays. (A) Immunofluorescence microscopy analysis of SIRT3 expression. SIRT3 (green) and nuclei (DAPI staining). Bar=20 μm. (B) Western blot analysis of SIRT3 expression. β-actin serves as loading control. (C-D) Clone formation assay. (C) Representative photographs of cell colonies; (D) Bar graph summarizing the number of colonies per field (*p < 0.05, Student’s t test). (E) Cell viability assay (**p < 0.01, two-way ANOVA, followed by post-hoc tests). (F-G) Soft agar assay. The number of tumor spheres per field was summarized in (F) (Bar=100 μm, **p < 0.01, one-way ANOVA followed by Tukey’s multiple comparison test) and representative images were shown with low (20X) and high (100X) magnifications in (G). (H-J) In vivo tumor formation assay of these cells. Tumor weights (*p < 0.05, one-way ANOVA followed by Tukey’s multiple comparison test) and tumor volumes (*p < 0.05, ***p < 0.001, two-way ANOVA followed by post-hoc test among the three groups) were summarized and photographs of dissected tumors at the end of experiments were shown. Data of Fig E, I (tumor volume) are expressed as means ±SD. Data of rest figures are expressed as means ±SEM.
ubiquitination level of c-MYC protein was increased in SIRT3 overexpressed cancer cells (Figure 4G). It has been reported that c-MYC phosphorylation at T58 was required for ubiquitination and degradation by proteasome [19, 25]. We analyzed the phosphorylation of c-MYC at T58 and observed marked increase in two prostate cancer cell lines after SIRT3 overexpression (Figure S4B). Together these data demonstrated that SIRT3 induced oncoprotein c-MYC destruction by stimulating ubiquitin-mediated proteolysis in prostate cancer cells.

SIRT3 destabilizes oncoprotein c-MYC level by regulating the PI3K-Akt pathway

The next question was to determine the signaling pathway(s) that underlay the regulation of c-MYC stability by SIRT3 in prostate cancers. Akt/mTOR pathway was highly activated in CRPC [26-28] and p-Akt was reported to target and stimulate the expression of tumorigenic factors like c-MYC [16], SOX2 [29, 30], OCT4 [31] and eventually robust tumor growth. SIRT3 has been reported to repress the activation of Akt in cardiac hypertrophy [32, 33], we therefore hypothesized that SIRT3 could inhibit oncoproteins via the Akt pathway in prostate cancer. Indeed, we found that the activation of Akt (p-Akt Ser473) was attenuated by SIRT3 overexpression both in vitro and in vivo (Figure 5A & 5B, and Figure S5). Conversely, p-Akt Ser473 was enhanced by SIRT3 silencing in prostate cancer cells (Figure S6A & S6B). These results indicated that SIRT3 suppressed the activation of Akt in prostate cancer cells.

To further investigate whether SIRT3 destructed oncoprotein c-MYC through the Akt pathway, we performed rescue experiments. In SIRT3 overexpressed prostate cancer cells we transfected a constitutively active, myristoylated form of Akt (Myr-Akt), or applied LY294002, a selective inhibitor of the PI3K/Akt pathway to inhibit p-Akt Ser473 signals [34] (Figure S7). The expression of c-MYC originally blocked by the overexpression of SIRT3 was up-regulated by the activation of Akt, and conversely c-MYC level could be further reduced by direct inhibition of PI3K/Akt pathway (Figure 5C & 5D). In addition, forced expression of constitutively active Akt attenuated the inhibitory effect of SIRT3 showing up-regulation of p-Akt Ser473 levels and increased colony formation, whereas inhibition of Akt signaling suppressed prostate cancer cell growth (Figures 5E & S8). Mechanistically, the ubiquitination level of c-MYC was reduced (Figure 5F) and degradation of c-MYC was attenuated (Figure 5G) by the activation of Akt in SIRT3 overexpressed prostate cancer cells. In addition, c-MYC phosphorylation is required for its ubiquitin-mediated degradation [19, 35]. We also noticed that the phosphorylation level at c-MYC T58 decreased upon Akt activation, corresponding to the lower ubiquitination level of c-MYC (Figure 5H).

SIRT3 suppresses PI3K/Akt pathway through down-regulating ROS level

SIRT3 is a mitochondria-located antioxidants trigger [9, 12] and ROS can activate the PI3K/Akt pathway [26, 36]. SIRT3 has also been reported to repress the activation of Akt in cardiac hypertrophy through ROS and relieve the disease symptoms [32, 33]. We hypothesized that SIRT3 might function by regulating the Akt pathway through ROS in prostate cancer. To test this, we measured ROS levels in three different CRPC lines. Overexpression of SIRT3 significantly suppressed the basal ROS levels (Figure 6A) while knockdown of SIRT3 increased ROS levels (Figure 6B). The activation of Akt (p-Akt Ser473) was also enhanced when SIRT3 was blocked (Figure S6). To explore whether SIRT3 could inhibit Akt phosphorylation at Ser473 by blocking ROS, we used antioxidant N-acetyl cysteine (NAC) to suppress the high ROS level in SIRT3 knockdown cancer cells (Figure 6C & 6D) and measured the p-Akt(Ser473) level concurrently. The p-Akt (Ser473) level was evidently reduced in the presence of NAC in SIRT3 silenced prostate cancer cells (Figure 6E & 6F). These data suggested that SIRT3 inhibited the activity of PI3K/Akt pathway by suppressing ROS in prostate cancer cells.

DISCUSSION

In this study, we have demonstrated for the first time that SIRT3 is moderately down-regulated in prostate cancers and patients with high SIRT3 copy number exhibit a significantly longer overall survival compared to those with low SIRT3 copy number. We have also shown that SIRT3 suppresses prostate cancer growth both in vitro and in vivo by inhibiting the activation of PI3K/Akt, thereby leading to the destruction of oncoprotein c-MYC. We show a SIRT3-Akt-c-Myc signaling axis that underlies the progression of prostate cancer (Figure 7), and suggest that: the molecular and pathological determination of SIRT3 may be used in prostate cancer diagnosis/prognosis. Strategies targeting this pathway may provide novel therapeutic options for prostate cancer.

It is crucial to point out that SIRT3 suppresses prostate cancer progression through the inhibition of PI3K/Akt pathway and eventually down-regulation and destruction of oncoprotein c-MYC. Such molecular mechanism underlying the suppression of tumor by SIRT3 has not been reported before. SIRT3 has been regarded as an aging-related protein and most studies of SIRT3 have focused on longevity [37-39]. SIRT3 was first reported as a tumor suppressor in breast carcinoma [12]. This mitochondrial tumor suppressor was later found to decrease hypoxia-inducible factor 1α (Hif1α) and genomic
Figure 4: SIRT3 induces oncoprotein c-MYC destruction in prostate cancer cells. (A) Relative mRNA expression levels of SIRT3 and c-MYC in prostate carcinoma (PCa) samples from selected Oncomine database (Luo2 Prostate Statistics, n=15). (B) Immunofluorescence microscopy of c-MYC and SIRT3 in prostate cancer cells. White arrows indicate cells with high SIRT3 expression and white triangle indicates cells with high c-MYC expression (Bar=10 μm). (C) Western blot and densitometry analysis of c-MYC expression between SIRT3 overexpressed group and control group in CRPC cell line C42B. (*p < 0.05, Student’s t test). (D) Immunohistochemical microscopy analysis of c-MYC in the frozen section of tumors formed from renal capsule model. (E) Analysis of c-MYC staining intensity in (D). (Bar=100 μm, *p<0.05, Student’s t test). (F) Western blot analysis of the degradation level of c-MYC between SIRT3 overexpressed group and control group in C42B treated with cycloheximide (CHX, 10 μg/ml). (***p < 0.001, one-way ANOVA followed by Tukey’s multiple comparison test). (G) Immunoprecipitation assay analysis of the ubiquitination level of c-MYC between SIRT3 overexpressed group and control group in C42B treated with the proteasome inhibitor MG132 (10 μM) for 8 h. Data are expressed as means ±SEM.
Figure 5: SIRT3 destabilizes oncoprotein c-MYC level by inhibiting PI3K/Akt pathway. (A) Western blot analysis of phospho-Akt (Ser473) and Akt levels in SIRT3 overexpressed group and control (Vector) group of prostate cancer cells. (B) Immunofluorescence staining of p-Akt in SIRT3 overexpressed cells and Vector control cells (Bar = 20μm). (C) and (D) Western blot analysis of c-MYC levels in SIRT3-overexpressed cells that were either transfected with Myr-Akt (vs. empty vector EV) or treated with PI3K-Akt pathway inhibitor LY294002 (LY 50 μM) vs. vehicle control (Ctrl). (E) Clone formation of the cancer cells mentioned in (C). (*p < 0.05, Student’s t test). (F) Immunoprecipitation assay analysis of the ubiquitination level of c-MYC in SIRT3-overexpressing C42B cells that transfected with Myr-Akt (vs. empty vector EV). (G) Western blot analysis of the degradation level of c-MYC in SIRT3-overexpressing C42B cells that transfected with Myr-Akt (vs. empty vector EV). (***p < 0.001, one-way ANOVA followed by Tukey’s multiple comparison test). (H) Western blot analysis and staining intensity analysis of p-c-MYC (T58) expression between SIRT3 overexpressed group and control group in C42B cell line. (***p < 0.01, Student’s t test). Data are expressed as means ±SEM.
Figure 6: SIRT3 suppresses PI3K-Akt pathway through down-regulating ROS levels. (A) and (B) Quantitative analysis of the ROS level in different prostate cancer cells up- or down-regulated with SIRT3 (*p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA followed by Tukey’s multiple comparison test). (C) and (D) Quantitative analysis of the ROS level in different SIRT3 knock out cancer cells treated with NAC (10μM) and vehicle control (*p < 0.05, **p < 0.01, one-way ANOVA followed by Tukey’s multiple comparison test). (E) and (F) Western blot analysis illustrated the p-Akt (Ser473) protein levels after these treatments. Data are expressed as means ±SEM.

Figure 7: A model summarizing the suppression of tumor progression by SIRT3 in prostate cancer as illustrated in this study.
instability, which led to cellular metabolic reprogramming and eventually limited carcinogenesis [13, 40]. However, whether SIRT3 played a role in regulating proto-oncogenes had remained unknown. Oncoproteins like c-MYC robustly drive tumor development and are key players in various types of cancers including prostate cancer [22, 24]. Herein we established a link between SIRT3 and c-MYC, which was connected by PI3K/Akt pathway. It has been shown that key signaling transduction pathways such as the Akt/mTOR pathway affect cancer cell survival and are highly activated in prostate cancer [26-28]. In addition, the activation of oncoprotein such as c-MYC is regulated by Akt pathway [22, 25]. Targeting c-MYC could put a brake on the rapid growth of cancer [35, 41, 42], but c-MYC is currently undruggable. Fortunately the fast rate of c-MYC protein degradation (half-life of ~30 min in rapidly dividing cells) allows tight regulation on c-MYC activity [43]. We here showed up- or down-regulation of SIRT3 affected c-MYC level and stability, which was mediated by the PI3K/Akt pathway. By resuming the activation of PI3K/Akt pathway, phosphorylation level at T58 of c-MYC decreased, leading to the reduction of the ubiquitin-mediated proteolysis of c-MYC. Our data suggest that controlling the activity of SIRT3 might be an alternative approach to manage c-MYC in cancer cells.

The Sirtuin family proteins play diverse roles in cancer development [44]. In addition to destabilizing c-MYC as reported herein, SIRT3 could function as a tumor suppressor possibly via its ability to deacetylate the proto-oncogene Skp2 [45]. Skp2 is overexpressed in a wide range of cancers including prostate cancer. In this regard, developing small molecules to activate SIRT3 might be a sound strategy for prostate cancer treatment. Our group has recently shown that a small molecule drug adjudin could efficiently up-regulate SIRT3 in normal cells [46]. Adjudin was also reported to be a potential anti-cancer drug as it targets proliferating cells [47]. To our best knowledge, no clinical trials have been reported using SIRT3 activator to treat cancer. As adjudin is generally regarded as a safe molecule [48, 49], future experiments should also be actively pursued and investigated for the treatment of prostate cancers.

**MATERIALS AND METHODS**

**Prostate cancer cell lines, plasmids and transfection**

Human prostate cancer cell lines DU145, PC3 and C42B were purchased from Shanghai Cell Collection (Shanghai, China) and maintained in basic Dulbecco’s modified Eagle’s medium (DMEM, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO). All cell lines were grown at 37°C in a humidified 5% CO₂ atmosphere. Full-length SIRT3 (plasmid #13814) and Myr-Akt (plasmid # 9005) plasmids were purchased from Addgene (www.addgene.org). Transfections were performed using jetPRIME (Polyplus) according to the manufacturer’s instructions.

**Construction lentiviral vectors and lentivirus production**

For lentivirus-based overexpression system, full-length human SIRT3 from Addgene (plasmid #13814) was cloned into a lentiviral vector under the control of CMV promoter. In brief, PCR was performed to obtain the SIRT3 cDNA using the primers 5'-CCGGAGATTCGACACATGGCGTTCTGGGG TTGGCGCAGGCCCGGAGC-3' and 5'-CTAGTCTAGAATATGACATTGCTGTTGTCCTCACAAGCTTTCCAGTTCCCG-3'. The PCR product was purified and subcloned into EcoRI and XhoI sites of the lentiviral pLVX-Neo-IRES vector (Biowit Company, www.biowit.com.cn). The pLVX-Neo-IRES vector without any sequence in MCS (multiple clone sites) was used as the control plasmid.

For lentivirus-based shRNA knockdown system, the specific target sequences of SIRT3 (sh1: 5'-CAACGTCACTCACTACTTT-3'; sh2: 5'-GGGTGCTTCAAGTGTTGTT-3') were cloned into the lentiviral shRNA vector under the control of U6 promoter. GV298 containing scrambled sequence was used as the control plasmid.

Lentivirus was produced by transfecting the packaging plasmids pCMV-dR8.74, pMD2.G (Addgene) as well as the transfer lentiviral plasmids into HEK-293T cells with calcium phosphate precipitation method. Medium containing lentivirus was harvested and filtered after 72h and 96 h of transfection. The lentivirus was concentrated from supernatants by ultracentrifugation and stocked at -80°C.

**Lentiviral infection**

Stable cell lines were generated by infection of cells with lentivirus, which was carried out in 24-well plate with serum-free DMEM medium. C42B, PC3 cells were transduced with lenti-SIRT3 at the infection MOI ≥90, and DU145, PC3 cells were transduced with lenti-sh-SIRT3 at the infection MOI ≥90 at 37°C with 8μg/ml polybrene for 24 h. Then culture medium with 10% FBS was replaced and cells were continuously cultured for 4 to 6 days followed by selection with G418 (Invitrogen) at500μg/ml.
Colony formation and soft agar assay

Clonogenic survival assays were performed by plating approximately 500-1,000 cells in 6-well culture dishes. In some experiments, cells were treated with vehicle (control) or treated with drugs. Cells were then fixed with 4% paraformaldehyde, stained with crystal violet solution and formed colonies (≥50 cells) were visually counted. Soft agar assay was performed in a six-well culture plate that was coated with 2 ml bottom agar mixture (DMEM with 10% FBS, 0.6% agar). After the bottom layer was solidified, 2 ml top agar-medium mixture (DMEM with 10% FBS, 0.3% agar) containing 10,000 cells were added, and the plate was incubated at 37°C for 2-3 weeks. Then the colonies were counted.

Cell proliferation assay

Cell viability was assessed by using Cell Titer 96® Aqueous One Solution Cell proliferation Assay kit (Promega, USA). This test is based on the change of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H tetrazolium), into a formazan product by NADPH or NADP in metabolically active cells. Medium was removed, washed with PBS. Then 20μl/well of Cell Titer 96® Aqueous One Solution was added and a 100μl/well of incubated solution medium was transferred to 96-well plate. After an hour, the optical density was read at 490 nm in a microplate reader (BioTek).

Immunohistochemistry and immunofluorescence microscopy

To examine the staining pattern of various target proteins in prostate cancer cells, the fixed preparations were first permeabilized in 0.5% Triton X-100 for 30 min, blocked with 10% normal goat serum at room temperature for 30 min, and then incubated with SIRT3(Cell Signaling), p-Akt (Ser473) (Cell Signaling), c-MYC (Epitomics), Ubiquitin (Santa Cruz), β-tubulin (Epitomics), β-actin (Epitomics) at 4 °C overnight and then hybridized with appropriate HRP-conjugated secondary antibody at room temperature for 1 h. Protein signals were visualized using ECL detection system (Thermo Scientific).

Western blot and immunoprecipitation assay

Cells were lysed in RIPA buffer (Millipore) supplemented with Complete Protease Inhibitor Cocktail (Roche), 2 mM PMSF, and 0.1% SDS. The protein concentration was measured using the BCA assay kit (Thermo Scientific). Total protein (~30 μg) was separated by 10% SDS-PAGE and then transferred to 0.45 μm nitrocellulose membrane (Millipore). The membrane was blocked with TBST containing 5% non-fat milk, incubated with SIRT3 (Cell Signaling), Akt (Cell Signaling), p-Akt Ser473 (Cell Signaling), c-MYC (Epitomics) at 4 °C overnight and then hybridized with appropriate HRP-conjugated secondary antibody at room temperature for 1 h. Protein signals were visualized using ECL detection system (Thermo Scientific).

For immunoprecipitation, 150-500 μg lysates prepared as above were incubated on ice with 4-10 μg of the appropriate antibodies and 20 μl protein G-sepharose beads (Roche) overnight. Immuno-complexes were washed 4-5 times with PBS (Invitrogen) before immunoblotted with the indicated antibodies.

Analysis of ROS production in cancer cells

The intracellular ROS level was measured by 2h,7′-dichlorofluorescein diacetate (DCFH-DA, Beyotime). In the presence of ROS, DCFH is converted into the highly fluorescent 2i,7′7acchlorofluorescein (DCF), which produces bright green fluorescence. Briefly, different prostate cells were cultured in a 24-well plate. After receiving various treatments, cells were washed twice in PBS and incubated with 10 μM DCFH-DA at 37°C in a humidified atmosphere of 95% air and 5% CO2. After 30 min, the extracellular ROS dye was washed away with DMEM without serum and the 24-well plate was then put in a microplate reader (BioTek) to measure the level of fluorescence. The excitation and emission wavelengths used were 482 nm and 535 nm, respectively.

Tumor formation assay in vivo

Male Balb/c nude mice of 3-5 weeks age (purchased from SLAC Laboratory, Shanghai) were maintained in pathogen-free conditions at animal facility of School of Biomedical Engineering, Shanghai JiaoTong University. For measuring the tumor formation ability of the various groups of prostate cancer cells, these cells were resuspended in serum-free medium and mixed with Matrigel at the ratio of 1:1, followed by subcutaneously injection into Balb/c nude mice (20,000cells per mouse). For measuring the tumor formation ability with renal
capsule model, SIRT3 overexpressed group and control group of cancer cells combined with murine urogenital sinus mesenchyme (UGSM) cells (1:1) were mixed with rat-tail collagen and grafted under kidney capsule of Balb/c nude mice. Tumor formation was evaluated once every three days after injection by palpation of injection sites. Between 2 weeks to 1 month, animals were killed and the tumor weights were measured. All studies were approved by the Institutional Animal Care and Use Committee, Shanghai Jiao Tong University, and all animals were treated in accordance with the institutional guidelines.

Statistical methods

The GraphPad Prism software was used in data processing and statistical analysis of significance. Data are presented as mean±SEM or mean±SD (If mentioned). Students’ t test was used to compare two groups (p < 0.05 was considered significant) and ANOVA with Tukey post-hoc test was used to compare three or more groups (p < 0.05 was considered significant).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES


46. Quan Y, Xia L, Shao J, Yin S, Cheng CY, Xia W and Gao WQ. Adjudin protects rodent cochlear hair cells against gentamicin ototoxicity via the SIRT3-ROS pathway. Scientific Reports. 2015; 5:8181.

