SIRT1 Inactivation Evokes Antitumor Activities in NSCLC through the Tumor Suppressor p27

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Abstract

p27kip1 (CDKN1B) regulates cellular proliferation and senescence, and p27kip1 deficiency in cancer is strongly correlated with poor prognosis of multiple cancer types. Understanding the mechanism of p27kip1 loss in cancer and the consequences of restoring p27kip1 levels is therefore critical for effective management during therapy. Here, SIRT1, a class III histone deacetylase (HDAC), is identified as an important regulator of p27kip1 expression. Mechanistically, SIRT1 reduces p27kip1 expression by decreasing p27kip1 protein stability through the ubiquitin–proteasome pathway. In addition, SIRT1 silencing suppresses non–small cell lung cancer (NSCLC) proliferation and induces senescence in a p27kip1-dependent manner. Furthermore, SIRT1 silencing dramatically suppresses tumor formation and proliferation in two distinct NSCLC xenograft mouse models. Collectively, these data demonstrate that not only SIRT1 is an important regulator of p27kip1 but also SIRT inhibition induces senescence and antigrowth potential in lung cancer in vivo.

Implications: SIRT1 is a key regulator of p27 protein levels and SIRT1 inhibition is a viable strategy for NSCLC therapy by means of p27 reactivation. Mol Cancer Res; 13(1); 41–49. ©2014 AACR.

Introduction

The p27kip1 protein is a member of a family of cyclin-dependent kinase (CDK) inhibitors (CDKIs) and plays an important role in multiple fundamental cellular processes and in the pathogenesis of human malignancies, with roles in cell proliferation, cell senescence, cell differentiation, migration, and invasion (1). p27kip1 is frequently inactivated in human cancers through accelerated proteolysis, and reduced p27kip1 expression has been shown to be associated with poor prognosis in a variety of human malignancies (2), whereas restoration of p27kip1 levels can inhibit tumor growth and progression (3, 4). Although it has been shown that p27kip1 expression is regulated primarily at the posttranscriptional level (5–7), the mechanism of p27kip1 reduction in cancer cells is still unclear. Identifying new pathways and molecules that regulate p27 expression is therefore critical for developing cancer treatments through restoration of p27 levels.

SIRT1 is a NAD-dependent non-histone and histone deacetylase (HDAC) that has been reported to play an important role in multiple cellular processes, such as p53, Foxo3, and E2F, have been identified as important regulators of cancer cell growth regardless of p53 status (10). p53 has been shown as an important target of SIRT1 in cancer growth and progression (14–19). In addition, several cellular proteins, such as p53, Foxo3, and E2F, have been identified to play an important role in SIRT1-mediated cancer cell survival (10). p53 has been shown as an important target of SIRT1 to suppress apoptosis (20); however, many studies show that SIRT1 regulates cancer cell growth regardless of p53 status (21–23). The specific mechanism of SIRT1 regulating cancer cell growth and survival is largely unknown.

Recently, several studies have indicated that p27kip1 may be a downstream target of SIRT1 (24, 25). In particular, it has been established that p27kip1 downregulation is consistently associated with poor prognosis in non–small cell lung cancer (NSCLC; refs. 26–28), and similarly, SIRT1 overexpression is significantly associated with unfavorable clinicopathologic factors, including high pathologic T-stage and lymph node metastasis in NSCLC (29, 30). Studies have also shown that SIRT1 inhibition suppresses NSCLC growth (25, 31–33), and SIRT1 knock-in in transgenic mice facilitates endothelial cell branching to increase vessel density and lung tumor growth (31). In addition, SIRT1 activation sensitizes lung cancer cells to the antitumor activity of cisplatin (34); in contrast, several other studies have shown that SIRT1 inhibition sensitizes lung and several other cancer types to the antitumor activity of cisplatin (19, 23, 30, 35–37). Despite these findings, it is largely unknown for the role of SIRT1 in NSCLC tumor growth and progression, and the relationship between SIRT1 and p27kip1 in the control of NSCLC growth and progression has not been established.

This study demonstrates for the first time that SIRT1 is a critical regulator of p27kip1 reduction in NSCLC; it is shown that SIRT1 promotes p27kip1 degradation through the ubiquitin–proteasome pathway. We find that SIRT1 silencing or inhibition suppresses NSCLC proliferation and induces senescence.
in NSCLC cells, and we demonstrate that p27kip1 is required for SIRT1 silencing-mediated cell senescence. Furthermore, we demonstrate that SIRT1 silencing dramatically suppresses NSCLC tumor formation in xenograft mouse models. Thus, we discover SIRT1 as a new regulator of p27kip1 degradation and identify a novel route of senescence induction in NSCLC cells.

Materials and Methods

Cell lines, plasmids, and antibodies

NSCLC cell lines, including H460, H1299, A549, H1703, H1975, HCC827, and H1993 cells, were obtained from ATCC. H1299 and H1703 cells were maintained in 1× RPMI-1640 plus 10% FBS; and H460, A549, H1975, HCC827, and H1993 cells were maintained in 1× DMEM plus 10% FBS. The human SIRT1 expression vector pCDNA3.1-SIRT1 and the pSUPER.retro.puro-SIRT1 were generated or acquired as previously described (38). Antibodies to p27 (sc-1641) and Ki67 (9027) were purchased from Santa Cruz Biotechnology. Antibodies to p27 (3686) and Ki67 (9027) were purchased from Cell Signaling Technology. Antibodies to SIRT1 (sc-74504, 1,000× dilution) were further incubated for 3 hours at room temperature with anti-SIRT1 (sc-74504, 1,000× dilution). Protein samples were subjected to Western blotting using p27 antibody (sc-1641, 1:400, CST) and then incubated with a biotin-conjugated anti-mouse or anti-rabbit antibody (1:400, CST) overnight. The tissue was embedded completely in histologic slides and kept at −80°C for 4 hours at room temperature.

Generation of SIRT1 or p27kip1 knockdown in an NSCLC cell line

Establishment of stable SIRT1-knockdown cell lines. The Phoenix packaging cell line was transfected with shRNA expression plasmids pSUPER.retro.puro-SIRT1#1 (5′-GATGACCGGCATGACGCTG-3′) or pSUPER.retro.puro-SIRT1#2 (5′-GATGACCGGCATGACGCTG-3′), or pSUPER.retro.puro.nontargeting shRNA control separately, using Lipofectamine 2000 (Invitrogen). After 48 hours, the medium containing retrovirus was collected, filtered, treated with polybrene, and transferred to H1299, H460, and A549 cell cultures. Infected cells were selected with puromycin, and the stably infected colonies were pooled.

p27 knockdown. Two p27 siRNA (On-Target plus SMART pool, #L-003472-00-00-05, Thermo Scientific Dharmacon, and SignalSilence p27 Kip I siRNA I, #12324, Cell Signaling Technology) or nontargeting siRNA control (# D-001810-01-20, Thermo Scientific Dharmacon) were transfected at 25 nmol/L with DharmaFECT transfection reagent to NSCLC for 72 hours, and the p27 levels were determined by immunoblot using p27 antibody.

Qualitative reverse transcription real-time PCR analysis

The qualitative reverse transcription real-time PCR (qRT-PCR) method has been previously described in detail (17). The primers used were p27 forward: CGCCCATATGGGACACTAA; p27 reverse: CGCGAGA-GCCGTCAGC-AA; β-actin forward: AAGGC-CACGGG-GAGAAGAT; β-actin reverse: CACAGCC-TGGGATC-CAAGTGACA. PCR reactions were performed in triplicate. The mRNA levels were normalized to β-actin. The specificities of the RT-PCR products were monitored by melt curve analysis.

Immunoblotting

Cells were lysed with 1% Nonidet P-40 lysis buffer (1% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 1 mmol/L Na2VO4) with protease inhibitor cocktail tablets (Cat#14586500, Roche Diagnostics). Protein samples were subsequently separated on a 12% or 8% SDS-PAGE and analyzed with anti-SIRT1 (sc-74504, 1,000×), anti-p27 (sc-1641, 500×), and anti-β-actin (A1987, 5,000×) antibodies.

In vivo experiments

The mouse work was performed at the Animal Core Facilities of the Boston University School of Medicine (Boston, MA) after approval by the Institutional Animal Care and Use Committee (IACUC). Briefly, SIRT1 (shRNA)-silenced or control (nontargeting shRNA) H1299 cells or H460 cells were diluted in 100 µL serum-free medium. The shRNA control cells were injected subcutaneously into the left flank of nude/nude male mice and the SIRT1-silenced cells were injected subcutaneously into the right flank of nude/nude male mice (Charles River Laboratories). The mice were observed every 3 days for the presence of tumors and the tumors were measured weekly with calipers. Each group contained 4 mice, and 2 groups of H1299 cells and 2 groups of H460 cells with independent SIRT1 silencing were studied. The tumor size was calculated on the basis of the formula: 0.52 × L × W × H.

SA-β-Gal analysis

The SIRT1-silenced and shRNA control NSCLC cell lines were seeded in 6-well plates (5 × 104 cells per well). After 3 days of culture, the cells were washed 3 times with 1× PBS and stained with X-gal solution (25 mg/mL X-gal in PBS buffer) overnight. The cells were observed under a microscope. Six or more separate fields were counted for each condition, and the percentage of SA-β-Gal–positive cells was calculated on the basis of 3 independent analyses.

Ki67 staining of frozen tissue sections

At the endpoint of the in vivo mouse study, the tumors were removed and fixed in 4% paraformaldehyde overnight at 4°C, then changed to 15% sucrose for 4 hours at room temperature. The tissue was embedded completely in optimal cutting temperature (OCT) compound and kept at −80°C. The tissue section was warmed at 37°C for 40 minutes on removal of the tissues section from the freezer and washed twice with PBS. The sections were further incubated for 3 hours at room temperature with Ki67 antibodies (1:400, CST) and then incubated with a biotin-conjugated secondary antibody followed by streptavidin–horseradish peroxidase with 3,3′-diaminobenzidine (DAB) as the substrate for immunodetection. Counterstaining was performed with hematoxylin. The detailed method followed the instructions of the Histostain Plus IHC Detection Kit (Invitrogen, #859673).

Results

SIRT1 negatively regulates p27kip1 expression

It was well-established that lower levels of p27kip1 correlate with poor prognosis of NSCLC (26, 39–41), and overexpression of SIRT1 also correlates with unfavorable clinicopathologic factors in NSCLC (29, 30). To study whether SIRT1 plays a role to lower p27kip1 expression in NSCLC cells, we treated NSCLC cells with various SIRT1 inhibitors to determine whether SIRT1 inhibition...
upregulates p27kip1 expression. SIRT1 inhibition by SIRT1 inhibitors, including Ex527, Sirtinol, and nicotinamide, was found to greatly upregulate p27kip1 levels in NSCLC cells (Fig. 1A and B). To more specifically study the role of SIRT1 in controlling p27kip1 levels, we knocked down SIRT1 in SIRT1-overexpressing NSCLC cells using SIRT1 shRNA to study the effect of SIRT1 silencing on p27kip1 expression. Consistent with the data generated by SIRT1 inhibitor treatment, SIRT1 silencing by highly specific genetic methods greatly upregulates p27kip1 expression (Fig. 1C and D). To further study the mechanism by which SIRT1 regulates p27kip1 expression, we performed qRT-PCR analysis to study whether SIRT1 regulates p27kip1 expression through regulating p27kip1 transcription. The p27kip1 mRNA level was unaffected by SIRT1 silencing (see Supplementary Fig. S1). These data suggest that SIRT1 plays an important role in p27kip1 down-regulation in NSCLC cells and that SIRT1-mediated regulation of p27kip1 protein expression does not take place at the level of transcription or alter mRNA stability.

SIRT1 regulates p27kip1 stability through the ubiquitin–proteolysis pathway

p27kip1 proteolysis plays a major role in controlling p27kip1 levels (7). We therefore sought to further determine whether increases in p27kip1 protein levels by SIRT1 silencing is due to changes in p27kip1 protein stability. The SIRT1 shRNA–silenced or nontargeting shRNA control NSCLC cells were treated with cycloheximide to inhibit protein synthesis, and the stability of p27kip1 protein between SIRT1-silenced and shRNA control cells was compared. The results show that the half-life of p27kip1 protein was dramatically increased in SIRT1-silenced cells compared with that in shRNA control cells (Fig. 2A and B). The p27 protein half-life was found to increase from 3.5 to 17 hours after SIRT1 silencing in H1299 cells (Fig. 2A) and to increase from 3.5 to 15 hours after SIRT1 knockdown in H460 cells (Fig. 2B). These data suggest that it is the alteration of p27kip1 protein stability that is the major cause for increased levels of p27kip1 protein expression in SIRT1-silenced cells.

Previous studies have shown that the ubiquitin–proteolysis pathway plays an important role in regulating p27kip1 protein levels (1). After finding that SIRT1 regulates p27kip1 stability, we next determined whether blocking proteasomal activity would reduce SIRT1-mediated p27kip1 downregulation. We ectopically expressed SIRT1 in H1299 cells, which were then treated with MG132 to block proteasome activity, and determined the effects of SIRT1 overexpression on p27kip1 expression. While SIRT1 overexpression reduced p27kip1 levels, blockade of proteasomal activity by MG132 produced a dramatic increase in the p27kip1 protein levels in SIRT1 ectopically expressed H1299 cells (Fig. 2C). This result suggests that blocking proteasomal activity by MG132 suppresses SIRT1-mediated p27kip1 degradation, which is consistent with our other data that show that SIRT1 regulates p27kip1 degradation through the ubiquitin–proteosome pathway.

SIRT1 inhibition/knockdown suppresses NSCLC proliferation

The role of p27kip1 in suppressing cell proliferation and inducing cell senescence is well known (1). After finding the importance of SIRT1 in controlling p27kip1 expression and stability, we further studied the role of SIRT1 in controlling NSCLC proliferation. We treated a panel of NSCLC cell lines with SIRT1 inhibitors Sirtinol and found that SIRT1 inhibition significantly decreases NSCLC proliferation in a dose-dependent manner (Fig. 3A). To validate the SIRT1 specificity of these results, we performed SIRT1 silencing by shRNA to study the effect of SIRT1 silencing on NSCLC proliferation. Responses similar to those of the SIRT1 inhibitor treatment experiment were seen, in that NSCLC proliferation was significantly suppressed by SIRT1 silencing (Fig. 3B).

SIRT1 silencing induces cell senescence through the p27kip1 pathway

To further study the mechanism by which SIRT1 suppresses NSCLC proliferation, we first performed a SA-β-Gal analysis in SIRT1-silenced and shRNA control NSCLC cells, including H460, H1299, and A549 cells. We detected much stronger SA-β-Gal positivity in SIRT1-silenced cells than in shRNA control cells (Fig. 3C). To validate the SIRT1 specificity of these results, we performed SIRT1 silencing by shRNA to study the effect of SIRT1 silencing on NSCLC proliferation. Responses similar to those of the SIRT1 inhibitor treatment experiment were seen, in that NSCLC proliferation was significantly suppressed by SIRT1 silencing (Fig. 3B).

Figure 1. SIRT1 regulates p27kip1 expression. A and B, SIRT1 inhibition with SIRT1 inhibitors upregulates p27 expression. H1299 (A) and H460 (B) cells were treated with Ex527 1 μmol/L, Sirtinol 100 μmol/L, or nicotinamide 10 mmol/L for 12 hours. Immunoblot analysis was performed with p27kip1 and β-actin antibodies. C and D, SIRT1 knockdown results in p27kip1 upregulation. H1299 (C) and H460 (D) cells were infected with SIRT1 shRNA or SIRT1 shRNA A2 or nonspecific shRNA control retrovirus, and SIRT1 stable knockdown colonies were collected. Cell extracts were made from SIRT1-silenced and shRNA control H1299 and H460 cells, and immunoblot analysis was performed with p27, SIRT1, and β-actin antibodies.
SIRT1 inactivation to induce senescence. To this end, we reduced the levels of p27Kip1 in SIRT1-silenced NSCLC cells using 2 independent p27Kip1-specific siRNA to study whether p27kip1 depletion would rescue the cell senescent phenotype induced by SIRT1 silencing. p27kip1 deletion in SIRT1-silenced cells greatly reduced the SA-beta-Gal staining compared with nontargeting siRNA-transfected control cells (Fig. 4B). This result demonstrates that p27kip1 is required for SIRT1 silencing–mediated cell senescence.

SIRT1 silencing exhibits strong antitumor activity in vivo

Because we have shown that SIRT1 inhibition or silencing induces cell senescence and inhibits NSCLC cell proliferation in vitro, we then examined the effect of SIRT1 silencing on suppression of tumor growth in vivo. We used H460 and H1299 NSCLC cell lines stably expressing either SIRT1-specific shRNA or a nontargeting control shRNA to determine their respective tendencies for tumor formation and growth in xenograft mouse models. Both the SIRT1-silenced and the shRNA control cells were injected into the same immunocompromised nu/nu mice to exclude individual variation (Fig. 5A and D). We performed 2 independent tumor injections with H1299 cells and 2 independent tumor injections with H460 cells, in which the SIRT1-silenced tumor cells were acquired using 2 specific SIRT1 shRNA expression vectors. We injected 4 mice each for the shRNA control group and for the SIRT1-silenced group. The results show that while 4 of 4 H460 control injected mice formed tumors, only 1 of 4 H460 SIRT1-silenced H460 mouse cells formed tumors; in addition, the tumorigenic growth of SIRT1-silenced cells was significantly retarded when compared with that of shRNA control cancer cells (Fig. 5B). Similar results were obtained from another independent H460 group: while 4 of 4 control-injected mice formed tumors, only 2 of 4 SIRT1-silenced H460 mouse cells formed tumors; in addition, the tumorigenic growth of SIRT1-silenced cells was significantly retarded when compared with that of shRNA control cancer cells (Fig. 5B). Similar results were obtained from another independent H460 group: while 4 of 4 control-injected mice formed tumors, only 2 of 4 SIRT1-silenced H460 mouse cells formed tumors; in addition, the tumorigenic growth of SIRT1-silenced cells was greatly reduced (Fig. 5C). In parallel with these H460 studies, we also performed 2 independent experiments with SIRT1-silenced and shRNA control H1299 cells. The tumor formation rate of control cells was 8 of 8 (100%), whereas the rate for SIRT1-silenced cells was 2 of 8, and the tumor volume of SIRT1 knockdown tumors was greatly
reduced compared with that of shRNA control H1299 cells (Fig. 5D–F). These data demonstrate that SIRT1 silencing has a dramatic effect on inhibiting lung tumor formation and growth in vivo in xenograft mouse models. At the endpoint of the mouse model experiment, we isolated the tumors and analyzed SIRT1 and p27kip1 levels by immunoblot analysis. The data show that small tumor size correlated with SIRT1 silencing and p27kip1 upregulation (Fig. 5G and H). We also show that SIRT1-silenced tumors had a lower proliferating cell fraction, as determined by IHC detection of Ki67 (Fig. 5I). Collectively, these results demonstrate that SIRT1 knockdown greatly reduces tumor formation and growth in NSCLC cells and that p27kip1 is an important downstream target of SIRT1.

Discussion

This study presents 3 new findings with important therapeutic implications. First, SIRT1 is a regulator of p27kip1 expression by controlling p27kip1 stability through the ubiquitin–proteolysis pathway, and SIRT1 inhibition can upregulate p27kip1 expression. Targeting SIRT1 could therefore be a novel strategy for moderating p27kip1 levels in cancer cells. Second, we show that SIRT1 silencing can induce cell senescence in NSCLC and that this response is dependent on p27kip1. Thus, we demonstrate a new route for senescence induction in established tumors. Third, we show that SIRT1 silencing profoundly suppresses NSCLC tumor formation and growth in vivo, and SIRT1 silencing–mediated growth suppression is associated with upregulated p27kip1 levels. Thus, our results suggest that SIRT1 inhibition could be a potential strategy for NSCLC treatment via its upregulation of p27kip1 expression.

SIRT1 induces p27-dependent, but p53- or p16-independent, cell senescence

Our data shows that SIRT1 regulates p27kip1 expression by controlling p27kip1 stability through the ubiquitin–proteolysis pathway, and SIRT1 silencing–mediated cell senescence depends on p27kip1. It has been established that cyclin inhibitors, including p27kip1, and the p16 and p53–p21 pathways, play an important role in cell senescence regulation (42, 43). Several studies have shown that SIRT1 inhibition increases p53 acetylation or upregulates p16 expression to induce cell senescence in endothelial cells or fibroblasts (44, 45). In NSCLC, a large percentage of cells contain either deleted or mutated p16...
or p53, whereas p27kip1 remains intact (46). In the NSCLC cells we studied, 5 of 7 cell lines had p53 deletion or mutation (Supplementary Table S1) and only 2 of 7 (H460 and A549) had wild-type p53. The p16 pathway was largely disrupted either through deletion (H460, A549, and H1993), methylation (H1299 and HCC827), or mutation (H1975; Supplementary Table S1). Our result shows that SIRT1 silencing can induce cell senescence in p53-deficient or p16-deficient cell lines, suggesting that the p53–p21 axis or p16 alone cannot be the only mediator of cell senescence after SIRT1 silencing. To test whether the p53–p21 pathway may be involved in SIRT1 silencing–mediated cell senescence in p53 wild-type cells, we checked the acetylated p53 and p21 levels in H460 cells. The results show that although SIRT1 silencing can increase acetylated p53 levels, the level of the p53-responsive gene p21 is downregulated instead of upregulated (Supplementary Fig. S3A). This is consistent with the report that adriamycin-induced p21 protein levels were not higher in SIRT1−/− and SIRT1+/−/− mouse embryonic fibroblasts (MEF) than in the corresponding WT and heterozygous MEFs (47), although there are other reports which show that p53 hyperacetylation in response to overexpression of a dominant-negative SIRT1 protein was accompanied by increased induction of the p53-responsive gene p21 (20). In addition to p53, our data also show that SIRT1 silencing can induce cell senescence in p16-deficient cell lines (H460 and A549; Supplementary Fig. S3B), indicating that p16 also does not play an important role in SIRT1 silencing–mediated cell senescence. Collectively, these findings suggest that p27kip1 rather than the p53–p21 pathway or p16 plays an important role in SIRT1 inhibition–mediated cell senescence. Thus, we have identified a new route for cell senescence induction through the SIRT1–p27 pathway in NSCLC cells.

SIRT1 inhibition is a potential consideration for NSCLC therapy

Cell senescence plays an important role in cancer development and progression (48), and therapy-induced senescence (TIS) has
emerged as a novel therapeutic approach for cancer eradication (42, 49, 50). Several TIS pathways have been identified that induce cell senescence and the eradication of established tumors, such as inactivation of MYC, SKP2, or CK2 (51, 52). Our data show that SIRT1 is a critical regulator of p27kip1 reduction in NSCLC cells, SIRT1 silencing induces cell senescence in a p27kip1-dependent manner, and also that SIRT1 silencing suppresses NSCLC tumor formation and growth in xenograft mouse models. Although NSCLC is a leading cause of cancer death, current treatment for advanced NSCLC is only moderately effective. Consistent with our findings, recent publications have shown that SIRT1 promotes NSCLC growth and progression, and SIRT1 inhibition induces antitumor activity in NSCLC (30, 31, 53). Moreover, SIRT1 overexpression has been shown to be significantly associated with poor prognosis of NSCLC (29, 30, 53, 54). Together with these results, our findings suggest that targeting the SIRT1–p27kip1 axis represents an important potential targeted strategy for treating NSCLC.

Figure 5.
SIRT1 knockdown dramatically suppresses NSCLC tumor formation and growth in vivo. The SIRT1-silenced and shRNA control H460 or H1299 cells were injected subcutaneously in the dorsal flanks of same athymic nude mice (the shRNA control cells is on the left side and SIRT1-silenced cells is on the right side). A, representative of H460-injected mouse (left, shRNA control H460; right, SIRT1-silenced H460). B, a total of $1 \times 10^6$ H460 SIRT1-silenced and shRNA control cells were injected and tumor volumes were measured at 28 to 34 days ($n = 4$). C, a total of $1.5 \times 10^5$ SIRT1-silenced and shRNA control H460 cells were injected and tumor volumes were measured at 21 to 27 days ($n = 4$). The SIRT1-silenced or shRNA control H460 cells used in B and C were generated from independent infections with 2 specific SIRT1 shRNA. D, representative of H1299-injected mouse (left, shRNA control H1299; right, SIRT1-silenced H1299). E, a total of $2 \times 10^5$ SIRT1-silenced and shRNA control H1299 cells were injected and tumor volumes were measured at 27 to 33 days ($n = 4$). F, a total of $1 \times 10^5$ SIRT1-silenced and shRNA control H1299 cells were injected and tumor volumes were measured at 40 to 46 days ($n = 4$). The SIRT1-silenced or shRNA control H1299 cells used in E and F were generated from independent infections with 2 specific SIRT1 shRNA. G, cell extracts were made from collected tumors of 2 independent H460 injections in group B and C, and immunoblot analysis was performed with p27, SIRT1, and $\beta$-actin antibodies. H, cell extracts were made from collected tumors of 2 independent H1299 injections in group E and F, and immunoblot analysis was performed with p27kip1, SIRT1, and $\beta$-actin antibodies. I, frozen sections of the collected tumor tissues from SIRT1-silenced and shRNA-control H460 and H1299 xenografts were stained with the proliferation marker Ki67.
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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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