

SIRT1 overexpression protects non-small cell lung cancer cells against osteopontin-induced epithelial-mesenchymal transition by suppressing NF- κ B signaling

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Abstract: Osteopontin (OPN) is a promoter for tumor progression. It has been reported to promote non-small cell lung cancer (NSCLC) progression via the activation of nuclear factor- κ B (NF- κ B) signaling. As the increased acetylation of NF- κ B p65 is linked to NF- κ B activation, the regulation of NF- κ B p65 acetylation could be a potential treatment target for OPN-induced NSCLC progression. Sirtuin 1 (SIRT1) is a deacetylase, and the role of SIRT1 in tumor progression is still controversial. The effect and mechanism of SIRT1 on OPN-induced tumor progression remains unknown. The results presented in this research demonstrated that OPN inhibited SIRT1 expression and promoted NF- κ B p65 acetylation in NSCLC cell lines (A549 and NCI-H358). In this article, overexpression of SIRT1 was induced by infection of SIRT1-overexpressing lentiviral vectors. The overexpression of SIRT1 protected NSCLC cells against OPN-induced NF- κ B p65 acetylation and epithelial-mesenchymal transition (EMT), as indicated by the reduction of OPN-induced changes in the expression levels of EMT-related markers and cellular morphology. Furthermore, SIRT1 overexpression significantly attenuated OPN-induced cell proliferation, migration and invasion. Moreover, overexpression of SIRT1 inhibited OPN-induced NF- κ B activation. As OPN induced NSCLC cell EMT through activation of NF- κ B signaling, OPN-induced SIRT1 downregulation may play an important role in NSCLC cell EMT via NF- κ B signaling. The results suggest that SIRT1 could be a tumor suppressor to attenuate OPN-induced NSCLC progression through the regulation of NF- κ B signaling.

Keywords: OPN, SIRT1, EMT, NF- κ B, NSCLC

Introduction

Lung cancer is one of the main reasons for cancer-related deaths worldwide.¹ Tumor metastasis is considered as the primary cause of mortality. Non-small cell lung cancer (NSCLC) is the dominant form of lung cancer, accounting for nearly 85% of the cases.² Study has indicated that more than 65% of patients show regional lymph node or distant site metastases when they were initially diagnosed with NSCLC.³ Therefore, it is necessary to explore the mechanisms regulating NSCLC metastasis for the development of potential new therapeutic targets. Epithelial-mesenchymal transition (EMT) is associated with multiple pathologies including lung cancer metastasis, during which epithelial cells acquire enhanced mobility and invasiveness by the loss of E-cadherin expression and the increase of mesenchymal marker (N-cadherin and Vimentin) expression.^{4,5} Further studies are needed to explore the molecular mechanism that regulates EMT, in order to find therapeutic target for the treatment of tumor invasion and metastasis.

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Osteopontin (OPN) is an extracellular matrix protein that plays a key role in tumor progression through binding with $\alpha\beta 3$ -integrin and CD44 receptor.⁶ The overexpression of OPN has been shown to correlate with poor prognosis in NSCLC.⁷ It has been demonstrated that OPN promotes EMT of several types of cancer cells, including endometrial cancer, prostate cancer, breast cancer and liver cancer.^{8–11} However, the mechanism underlying OPN-induced EMT remains poorly understood. Nuclear factor- κ B (NF- κ B) is a nuclear transcription factor that stimulates the expression of transcription factors that drive the EMT process. It has been shown to be involved in OPN-induced tumor progression.^{12–14} It has been shown that the acetylation of RelA/p65, a subunit of NF- κ B, can increase its specific transcriptional activity and the deacetylation will inhibit its transactivation.^{15,16} Therefore, it can be inferred that deacetylation of NF- κ B p65 could be a potential target to suppress OPN-induced NSCLC cell EMT. However, the acetylation level of NF- κ B p65 in OPN-induced EMT remains unclear.

Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide-dependent lysine deacetylase.¹⁷ The role of SIRT1 in tumor progression is still controversial. Initially SIRT1 was shown to suppress apoptosis by deacetylation of p53, a well-known tumor suppressor.¹⁸ However, SIRT1 is regarded as a tumor suppressor that inhibits tumor progression by targeting HIF-1 α , TGF- β /Smad4 or NF- κ B/cyclin D1 signaling pathway.^{19–21} In addition, resveratrol, the SIRT1 activator, has been shown to activate caspase-3 and reduce chemoresistance in breast tumor cells through the inhibition of NF- κ B-specific transcriptional activation.²² However, little is known regarding the role of SIRT1 as regulator of NF- κ B activation during EMT process. SIRT1 inhibits NF- κ B activation by deacetylation of the RelA/p65 subunit at lysine 310.¹⁶ Therefore, we examined the effect of OPN on SIRT1 expression and the role of SIRT1 in OPN-induced EMT process.

This study aimed to examine the mechanisms of OPN-induced EMT in NSCLC. It is found that OPN inhibits SIRT1 expression, and SIRT1 protects against OPN-induced EMT by inhibiting NF- κ B signaling activation.

Materials and methods

Cell culture and reagents

NSCLC cell lines A549, NCI-H358 (H358), NCI-H1299 (H1299) and NCI-H460 (H460) and normal lung epithelium cell line BEAS-2B were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, People's Republic of China) and were cultured in RPMI 1640

(HyClone, Logan, UT, USA) supplemented with a mixture of 10% fetal bovine serum (FBS) and antibiotics at 37°C in an incubator filled with 5% CO₂. Recombinant human OPN protein (rhOPN) was purchased from R&D Systems (Minneapolis, MN, USA).

Enzyme linked immunosorbent assay

A549, NCI-H358, NCI-H1299, NCI-H460 and BEAS-2B cell lines were seeded in T25 culture bottle at a density of 3×10^6 /bottle; cell culture supernatants were collected after cells were cultured for 24 hours. The secretory levels of OPN in these cells were detected using a human OPN enzyme-linked immune detection kit according to the manufacturer's instructions (R&D Systems).

Cell infection

Lentiviral vectors overexpressing SIRT1 (LV-SIRT1) and their negative control (LV-NC) were constructed by GeneChem Co., Ltd (Shanghai, People's Republic of China) and used to infect A549 and NCI-H358 cell lines. For infection, cells were plated in 6-well plates at a density of 1×10^5 cells/well. After 24 hours, the cells were infected with 5 μ L of the lentiviral vector by using polybrene (8 μ g/mL) according to the manufacturer's instructions. At 96 hours after infection, the effect of the infection was evaluated by real-time quantitative polymerase chain reaction (qPCR) and Western blot analysis.

CCK-8 assay

Cell suspension (5×10^4 /mL) was seeded in 96-well plate (100 μ L per well). Cells were cultured overnight at 37°C in an incubator filled with 5% CO₂. The cells infected with LV-SIRT1 and LV-NC were treated with different concentrations of OPN (0, 100, 200 and 400 ng/mL) for 24 hours or treated with 400 ng/mL OPN for various times (0, 12, 24 and 48 hours). The concentration of FBS in culture medium was reduced to 5%. To detect cell proliferation, cells were incubated with 10 μ L Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) for 2 hours in an incubator at 37°C. The absorbance was measured at a wavelength of 450 nm.

Real-time PCR (quantitative PCR)

Total RNA was extracted from NSCLC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the PrimeScript™ RT reagent Kit (Takara, Dalian, People's Republic of China). Aliquots of the resulting cDNA were used for PCR amplification in Roche light cycler 480 (Roche Diagnostics GmbH, Basel, Switzerland) using the SYBR Green Supermix (Takara). The specific primer sequences

used to determine the mRNA expressions were as follows: SIRT1 F: 5'-TAGCCTTGTCAGATAAGGAAGGA-3', R: 5'-ACAGCTTCACAGTCAACTTTGT-3'; OPN F: 5'-CGAGGAGTTGAATGGTGCATAC-3', R: 5'-TTTCAGCACTCTGGTCATCCA-3'; Vimentin F: 5'-ACCGCTTTGCCAACTACAT-3', R: 5'-TTGTCCCGCTCCACCTC-3'; E-cadherin F: 5'-ACCATTAACAGGAACACAGGAG-3', R: 5'-GGCATCAGCATCAGTCACTT-3'; N-cadherin F: 5'-CATCATCATCCTGCTTATCCTTGT-3', R: 5'-TTCTCCTCCACCTTCTTCATCATA-3'; GAPDH F: 5'-GAGTCAACGGATTTGGTCGT-3', R: 5'-GACAAGCTTCCCGTTCTCAG-3'. GAPDH was used as an internal control. The relative expressions of the genes were quantified using the $2^{-\Delta\Delta CT}$ method.

Western blot

Total protein was extracted from cells on ice using RIPA lysing buffer. The nuclear and cytoplasmic proteins were separated by nuclear and cytoplasmic protein extraction kit (Beyotime, Jiangsu, People's Republic of China) according to the manufacturer's instructions. The protein content was quantified by using the BCA protein assay kit (Beyotime). Samples containing 40 μ g proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA), which was blocked and incubated overnight at 4°C with specific primary antibodies against SIRT1 (Abcam, Cambridge, UK), acetyl NF- κ B p65 Lys310 (Abcam), E-cadherin (Proteintech, Rosemont, IL, USA), N-cadherin (Proteintech), Vimentin (Proteintech), NF- κ B p65 (Abcam), IKK- α (Cell Signaling Technology, Danvers, MA, USA), I κ B- α (Cell Signaling Technology), Lamin B (Proteintech) and β -actin (Proteintech). The membrane was washed and incubated with HRP-conjugated secondary antibody (Proteintech) at room temperature for 90 minutes. After washing the membrane, signals were detected using an ECL kit (Thermo; Rockford, IL, USA).

Wound healing migration assay

The cells (infected with either LV-SIRT1 or LV-NC) were plated onto 6-well plates and then cultured to 90% confluence. Cell monolayers were carefully wounded by scratching with the 200 μ L pipette tip. The cells were then washed twice with PBS to remove dislodged cells and cultured in serum-free RPMI 1640 culture medium with either control buffer (CB) or 400 ng/mL OPN for 48 hours. Wound closure rate = (wound width at 0 hour – wound width at 48 hours)/wound width at 0 hour \times 100%.

Transwell invasion assay

Cells (infected with either LV-SIRT1 or LV-NC) were treated with either CB or 400 ng/mL OPN for 48 hours. The cells in serum-free medium were added to the upper chambers (8×10^4 cells/well) of a 24-well transwell chamber (8 μ m, Corning Life Sciences, Corning, NY, USA) coated with 40 μ L Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) diluted 1:8 in serum-free medium. The chambers were incubated for 48 hours with 600 μ L complete culture medium, which contained 15% FBS added in the lower chamber. Non-invading cells were removed from the upper chamber with cotton swabs. Cells that had invaded through membrane on the lower surface of the membrane were fixed and stained with crystal violet (Beyotime). The membranes were captured with digital images, and the number of invading cells on the filters were counted in random fields.

Immunofluorescence

Cells (infected with either LV-SIRT1 or LV-NC) were cultured in 12-well plates containing coverslips, treated with CB or OPN (400 ng/mL) for 2 hours, and then washed with PBS. The cells were fixed in 4% paraformaldehyde for 30 minutes and permeabilized in 0.1% Triton X-100 solution. Cells were blocked with goat serum for 1 hour and incubated overnight at 4°C with primary antibody against NF- κ B p65 (Abcam). Subsequently, cells were washed three times with PBS, followed by incubation with FITC-conjugated anti-rabbit antibody (Proteintech) at room temperature for 3 hours. The cell nuclei were stained with DAPI and the cells were observed by fluorescence microscopy.

Statistical analysis

All of the experiments were repeated at least three times for statistical analysis. All data would be presented as mean \pm SD. Student's *t*-test was used to compare data between two groups. $P < 0.05$ was considered statistically significant.

Results

OPN is highly expressed by NSCLC cells and promotes NSCLC cells EMT

Real-time qPCR revealed that the mRNA expression levels of OPN in human NSCLC cell lines (A549, NCI-H358, NCI-H1299, NCI-H460) were higher than that in normal lung epithelium cell line BEAS-2B (Figure 1A). Consistent with this, enzyme linked immunosorbent assay revealed that the protein secretion levels of OPN in these NSCLC cell lines were higher than that in normal lung epithelium cell line (Figure 1B). In these cells, the protein secretion levels

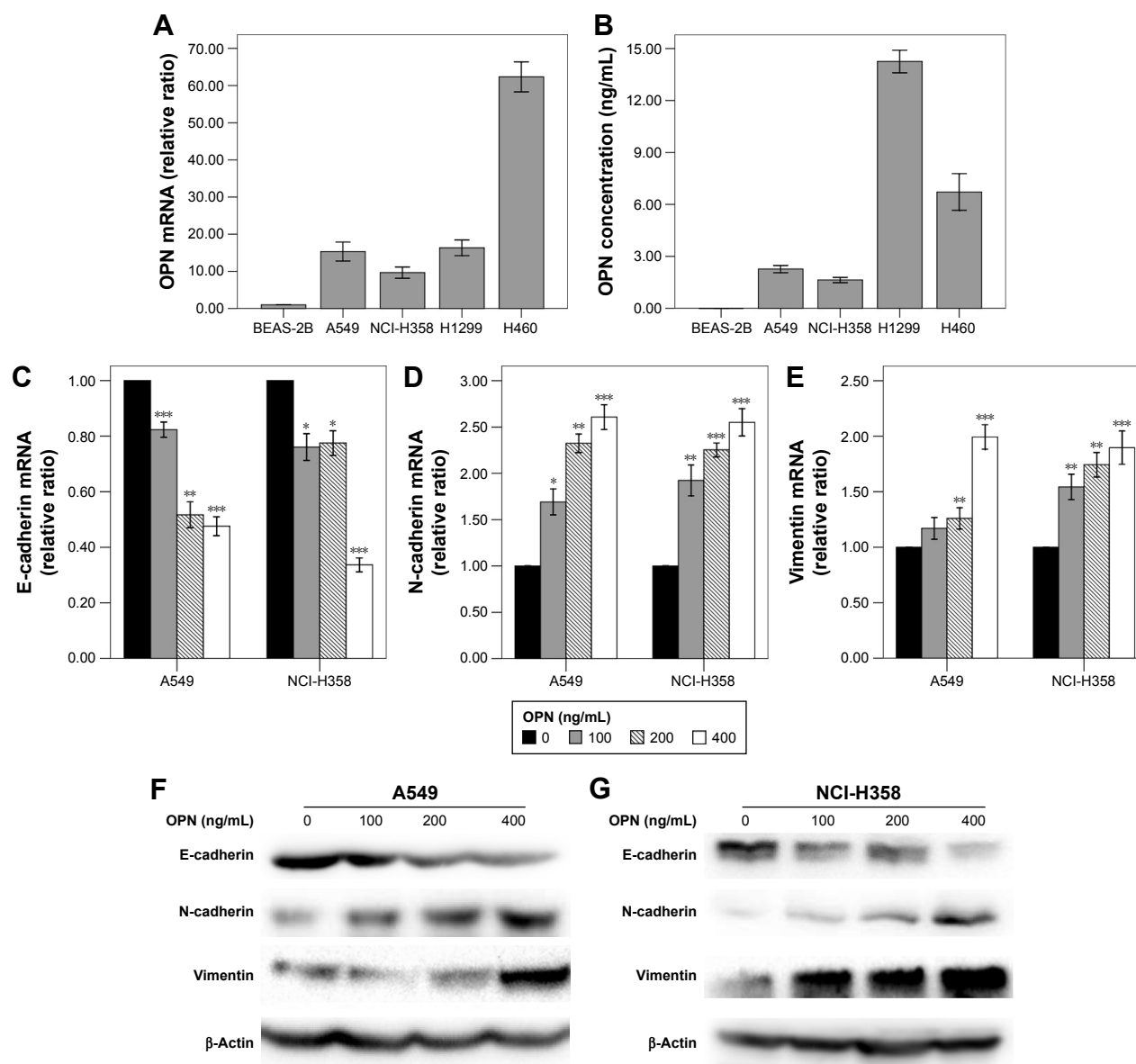


Figure 1 OPN is highly expressed by NSCLC cells and promotes NSCLC cell EMT.

Notes: (A) The mRNA expression levels of OPN in NSCLC cell lines (A549, NCI-H358, H1299, H460) and normal lung epithelium cell line (BEAS-2B) were examined by real-time qPCR. GAPDH was used as an internal control. The mRNA expression of OPN measured in BEAS-2B is presented as 1. (B) The protein secretion levels of OPN were examined by ELISA. The detection range of the kit is 0.3–90 ng/mL. (C–E) Real-time qPCR was used to examine the mRNA levels of E-cadherin, N-cadherin and Vimentin in A549 and NCI-H358 cells treated with an increasing concentration of OPN (0, 100, 200 and 400 ng/mL) for 24 hours. GAPDH was used as an internal control. The mRNA expression of target genes in control group is presented as 1. Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control. (F and G) Western blot was used to detect the protein levels of E-cadherin, N-cadherin and Vimentin in A549 and NCI-H358 cells treated with an increasing concentration of OPN (0, 100, 200 and 400 ng/mL) for 24 hours. β -Actin was used as an internal control. The group treated with control buffer served as control.

Abbreviations: OPN, osteopontin; NSCLC, non-small cell lung cancer; EMT, epithelial-mesenchymal transition; ELISA, enzyme linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; SD, standard deviation.

of OPN in A549 and NCI-H358 cells were lower than that in NCI-H1299 and NCI-H460 cells (Figure 1B). Therefore, A549 and NCI-H358 cells were treated with rhOPN for further research. Real-time qPCR and Western blot analyses revealed that incubation with OPN for 24 hours resulted in a decrease of epithelial marker E-cadherin expression and an increase of mesenchymal markers N-cadherin and Vimentin expression. The changes of EMT marker expression in cells treated with 400 ng/mL OPN were the most significant at

both mRNA and protein levels, which suggests that OPN significantly promotes EMT of A549 and NCI-H358 cells (Figure 1C–G and Figure S1A–C).

OPN inhibits SIRT1 expression and promotes acetylation of NF- κ B p65 subunit

Firstly, the SIRT1 expression levels were examined in response to OPN. Real-time qPCR and Western blot analyses revealed

that incubation with OPN (0, 100, 200 and 400 ng/mL) for 24 hours resulted in a decrease of SIRT1 expression in A549 and NCI-H358 cells (Figure 2A–D). As SIRT1 can inhibit the activation of NF- κ B by deacetylation of the RelA/p65 subunit

at lysine 310,¹⁶ the acetylation of RelA/p65 was evaluated after treatment with OPN for 24 hours. Figure 2B, C and E showed that OPN significantly increased the protein levels of acetyl NF- κ B p65; this is consistent with the decrease of

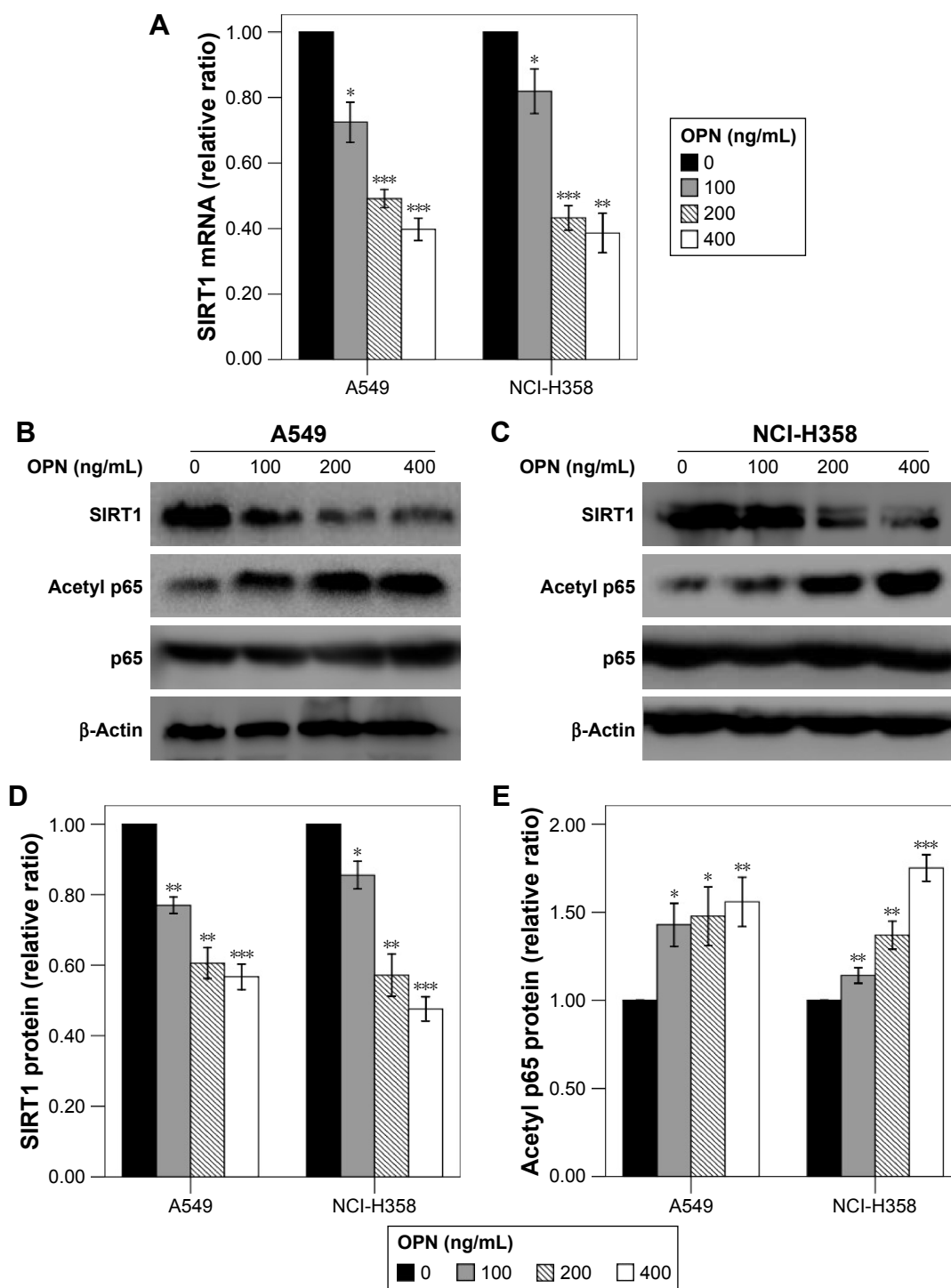


Figure 2 OPN inhibits SIRT1 expression and promotes acetylation of NF- κ B p65 subunit.

Notes: (A) Real-time qPCR was used to detect the mRNA expression of SIRT1 in A549 and NCI-H358 cells treated with an increasing concentration of OPN (0, 100, 200 and 400 ng/mL) for 24 hours. GAPDH was used as an internal control. (B–E) Western blot was used to detect the protein expression of SIRT1, acetyl NF- κ B p65 and NF- κ B p65 in A549 and NCI-H358 cells treated with an increasing concentration of OPN (0, 100, 200 and 400 ng/mL) for 24 hours. β -Actin was used as an internal control. The group treated with control buffer served as control. The mRNA and protein expression of target genes in control group is presented as 1. Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control.

Abbreviations: OPN, osteopontin; SIRT1, Sirtuin 1; NF- κ B, nuclear factor- κ B; qPCR, quantitative polymerase chain reaction; SD, standard deviation.

SIRT1 expression in A549 and NCI-H358 cells. At the same time, the protein expression levels of NF- κ B p65 were not significantly changed by OPN treatment.

SIRT1 overexpression inhibits OPN-induced NSCLC cell EMT

In order to examine the role of SIRT1 in OPN-induced EMT, NSCLC cells (A549 and NCI-H358) were infected with LV-SIRT1 or LV-NC. After 96 hours, infection efficiency was determined by real-time qPCR and Western blot analysis. SIRT1 expression was significantly increased in cells infected with LV-SIRT1 (Figure S2A–C). Cells (infected with either LV-SIRT1 or LV-NC) were stimulated with 400 ng/mL OPN. The cells infected with LV-NC followed by treatment with OPN for 72 hours exhibited mesenchymal fibroblast-like morphology. By contrast, SIRT1-overexpressing cells treated with OPN exhibited epithelial cobblestone-like appearance (Figure S2D). These morphological changes suggested that the overexpression of SIRT1 in NSCLC cells may reverse OPN-induced EMT. Furthermore, the expression changes of E-cadherin, N-cadherin and Vimentin in SIRT1-overexpressing cells followed by treatment with OPN (400 ng/mL) for 24 hours were examined. The results demonstrated that SIRT1 overexpression attenuated OPN-induced downregulation of E-cadherin and upregulation of N-cadherin and Vimentin at both mRNA and protein levels (Figure 3A–H). All these results indicated that OPN promotes NSCLC cell EMT, at least in part, by the inhibition of SIRT1 expression. At the same time, SIRT1 overexpression inhibited OPN-induced NF- κ B p65 acetylation (Figure 3D, E and I). However, neither SIRT1 overexpression nor OPN treatment could influence NF- κ B p65 protein expression levels (Figure 3D and E).

SIRT1 overexpression inhibits OPN-induced NSCLC cell proliferation

The effect of SIRT1 on OPN-induced NSCLC cell proliferation was assessed. A549 and NCI-H358 cells were infected with LV-SIRT1 or LV-NC followed by treatment with different concentrations of OPN (0, 100, 200, 400 ng/mL) for 24 hours. CCK-8 assays were, then, performed on A549 and NCI-H358 cells. As shown in Figure 4A and B, compared with untreated cells, OPN promoted A549 and NCI-H358 cell proliferation. However, in SIRT1 overexpression cells, OPN could not significantly promote cells proliferation. A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with CB or 400 ng/mL OPN for different times (0, 12, 24, 48 hours). CCK-8 assays showed that SIRT1

overexpression inhibited OPN-induced A549 and NCI-H358 cell proliferation at 12, 24 and 48 hours after OPN treatment (Figure 4C and D).

SIRT1 overexpression inhibits OPN-induced NSCLC cell migration and invasion

The roles of SIRT1 in OPN-induced NSCLC cell migration and invasion were explored using wound healing assay and transwell assay, respectively. Wound healing assay showed that OPN significantly increased the migration rate of the NSCLC cell lines A549 and NCI-H358 (Figure 5A and B). The overexpression of SIRT1 in A549 and NCI-H358 cells significantly inhibited OPN-induced cell migration (Figure 5A and B). Similarly, OPN promoted cell invasion, and overexpression of SIRT1 significantly depressed OPN-induced cell invasion (Figure 5C). This phenomenon indicated that SIRT1 is involved in inhibition of OPN-induced NSCLC cell migration and invasion.

SIRT1 overexpression inhibits OPN-induced NF- κ B signaling activation

NF- κ B is an important signaling pathway that mediates OPN-induced tumor progression. So, acetyl NF- κ B p65 protein expression level was explored in cells treated with OPN, revealing that OPN promoted acetyl NF- κ B p65 protein expression (Figure 2B, C and E). Furthermore, SIRT1 overexpression inhibited OPN-induced NF- κ B p65 acetylation (Figure 3D, E and I). Therefore, the assumption, where OPN promotes NF- κ B p65 subunit activation through inhibiting SIRT1 expression was explored. The role of SIRT1 in OPN-induced NF- κ B activation was explored using Western blot and immunofluorescence assay in A549 and NCI-H358 cells. The analysis from the Western blot clearly showed that OPN increased the nuclear translocation of the NF- κ B p65 subunit and hence OPN-induced NF- κ B activation in comparison to untreated cells (Figure 6A). However, OPN-induced NF- κ B p65 nuclear translocation was greatly inhibited by the overexpression of SIRT1 (Figure 6A). Similarly, immunofluorescence assay also showed that in both untreated cells and SIRT1-overexpressing cells treated with OPN, NF- κ B p65 subunit was mainly located in the cytoplasm. However, in cells treated with OPN alone, NF- κ B p65 was translocated to the nucleus (Figure 6B). Furthermore, the effect of OPN on the upstream molecules involved in NF- κ B activation was examined. Treatment of A549 and NCI-H358 cells with OPN caused a degradation of I κ B- α and an increase in the expression of IKK- α (Figure 7A). SIRT1 overexpression inhibited

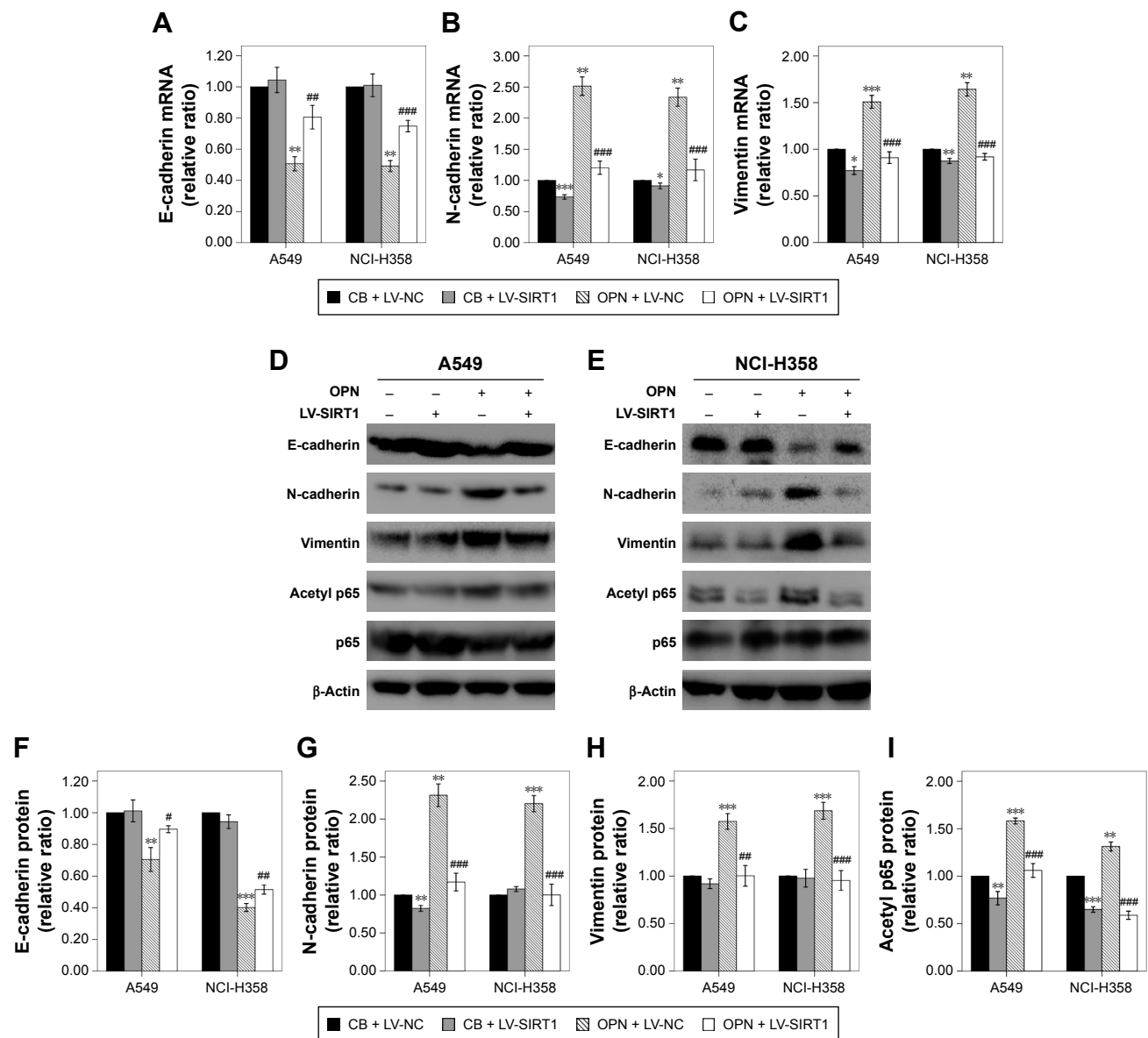


Figure 3 Overexpression of SIRT1 inhibits OPN-induced EMT and NF- κ B p65 acetylation.

Notes: (A–C) A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with OPN (400 ng/mL) for 24 hours, and the mRNA expression levels of E-cadherin, N-cadherin and Vimentin were determined by real-time qPCR analysis. GAPDH was used as an internal control. (D–I) A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with OPN (400 ng/mL) for 24 hours, and the protein expression levels of E-cadherin, N-cadherin, Vimentin, acetyl NF- κ B p65 and NF- κ B p65 were determined by Western blot. β -Actin was used as an internal control. The group treated with CB + LV-NC served as control. The mRNA and protein expression of target genes in control group is presented as 1. Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 versus OPN + LV-NC.

Abbreviations: SIRT1, Sirtuin 1; OPN, osteopontin; EMT, epithelial-mesenchymal transition; NF- κ B, nuclear factor- κ B; LV-SIRT1, lentiviral vectors overexpressing SIRT1; NC, negative control; qPCR, quantitative polymerase chain reaction; CB, control buffer; SD, standard deviation.

OPN-induced downregulation of I κ B- α and upregulation of IKK- α (Figure 7A). These results indicated that OPN induces NF- κ B signaling activation through the inhibition of SIRT1 expression. In order to examine the role of NF- κ B signaling in OPN-induced NSCLC cell EMT, A549 and NCI-H358 cells were pretreated with NF- κ B inhibitor (PDTC, 10 μ M) for 30 minutes followed by treatment with OPN (400 ng/mL) for 24 hours. Western blot showed that PDTC significantly inhibited OPN-induced decrease of E-cadherin expression and

increase of N-cadherin expression (Figure 7B). In conclusion, OPN promoted NSCLC cell EMT by activating NF- κ B signaling, and SIRT1 overexpression inhibited OPN-induced EMT by inhibiting NF- κ B signaling activation.

Discussion

Previous studies indicated that OPN expression varies significantly in non-carcinomatous and carcinomatous tissues and it is a potential biomarker in various types of cancer,

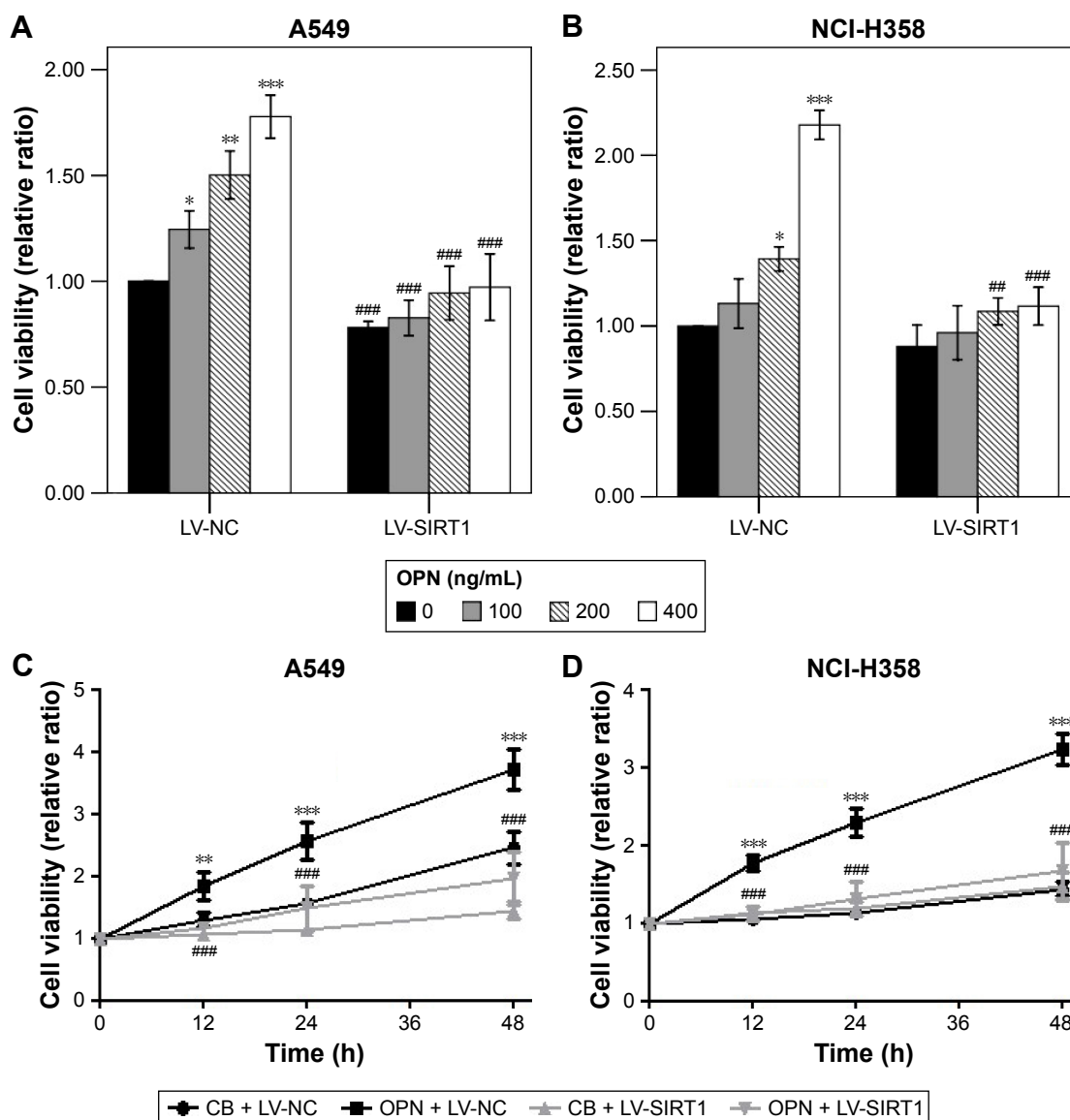


Figure 4 Overexpression of SIRT1 inhibits OPN-induced cell proliferation.

Notes: (A and B) A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with OPN (0, 100, 200, 400 ng/mL) for 24 hours; cell proliferation was examined by CCK-8 assay. The group treated with CB + LV-NC served as control. The absorbance in control group is presented as 1. (C and D) A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with OPN (400 ng/mL). Cell viability was examined by CCK-8 assay at 0, 12, 24, 48 hours after OPN treatment. The absorbance at 0 hour serving as control is presented as 1. Results presented are mean \pm SD analyzed with Student's t-test from at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ OPN + LV-NC versus CB + LV-NC. ### $P < 0.01$, #### $P < 0.001$ OPN + LV-SIRT1 versus OPN + LV-NC.

Abbreviations: SIRT1, Sirtuin 1; OPN, osteopontin; LV-SIRT1, lentiviral vectors overexpressing SIRT1; NC, negative control; CCK-8, Cell Counting Kit-8; CB, control buffer; SD, standard deviation.

including gastric cancer,²³ liver cancer,²⁴ bladder urothelial cancer,²⁵ prostate cancer,²⁶ breast cancer²⁷ and NSCLC.²⁸ OPN is involved in multiple tumor-associated biological processes, including the induction of tumor cell immune evasion, survival, invasion, metastasis and angiogenesis.²⁹ The EMT process appears to be important for tumor cells to acquire multiple drug resistance, an increased migration and invasion ability, and cancer stem cells characteristics.⁵ In our study, NSCLC cell lines were shown to be with a

higher OPN expression than normal lung epithelium cell line, which is consistent with previous studies.

In this study, we found that OPN-induced EMT in NSCLC cells may depend, at least partially, on the down-regulation of SIRT1. SIRT1 overexpression attenuated OPN-induced NSCLC cell migration and invasion via inhibition of EMT. Previously, it has been shown that SIRT1 regulates various physiological processes by the inhibition of NF- κ B signaling pathway as a deacetylase.^{22,30,31} Here,

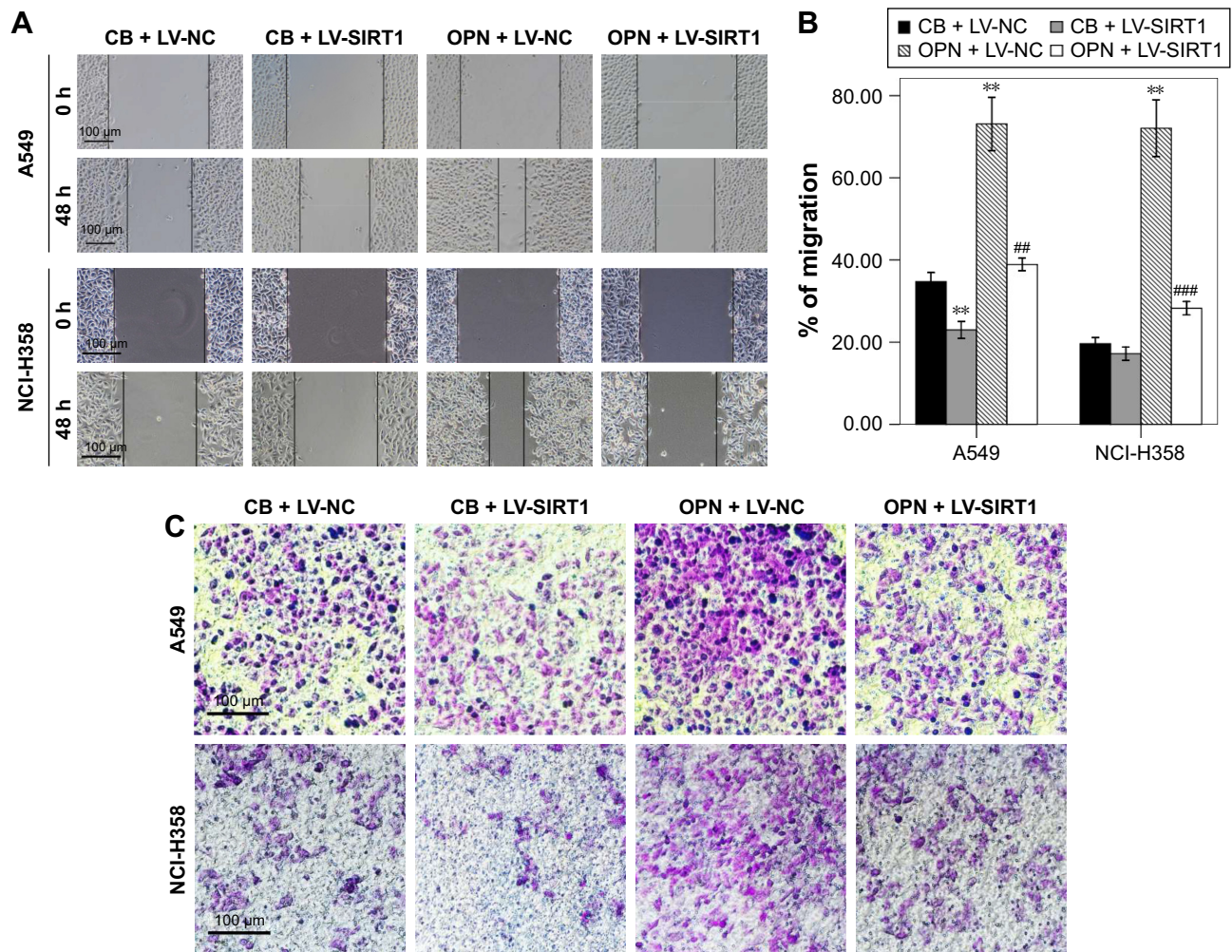


Figure 5 Overexpression of SIRT1 inhibits OPN-induced cell migration and invasion.

Notes: (A and B) A549 and NCI-H358 cells were infected with LV-SIRT1 or LV-NC followed by stimulation with OPN (400 ng/mL). Cell migration was examined by wound healing assay. Representative images at 48 hours after scratching are presented ($\times 100$). Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. ** $P < 0.01$ versus CB + LV-NC. ## $P < 0.01$, ### $P < 0.001$ versus OPN + LV-NC. (C) A549 and NCI-H358 cells were infected with LV-SIRT1 or LV-NC followed by stimulation with OPN (400 ng/mL) for 48 hours. Cell invasion was examined by transwell assay. After incubation for 48 hours, the cells that had invaded to the lower chamber were fixed, stained and counted. Representative images are presented ($\times 100$). Scale bars represent 100 μ m.

Abbreviations: SIRT1, Sirtuin 1; OPN, osteopontin; LV-SIRT1, lentiviral vectors overexpressing SIRT1; NC, negative control; SD, standard deviation.

the results showed that treatment of A549 and NCI-H358 cells with OPN increased the expression of acetyl NF- κ B p65, while SIRT1 overexpression attenuated OPN-induced NF- κ B p65 acetylation. Furthermore, it is demonstrated that SIRT1 overexpression inhibited OPN-induced NF- κ B p65 signaling activation. The results showed that SIRT1 may protect NSCLC cells against OPN-induced EMT by inhibiting NF- κ B activity. It is well known that the EMT process plays an important role in the transition of lung cancer from early stage to invasive carcinoma.⁴ Taken together, these data suggested that SIRT1 might be a new therapeutic modality in OPN-mediated NSCLC progression.

It is worth noting that previous studies have indicated that SIRT1 exerted both stimulative^{32–34} and inhibitory

effects^{20,35,36} on tumor cell EMT. In prostate cancer, SIRT1 has been reported to induce EMT by cooperating with EMT-related transcription factor ZEB1.³² Moreover, in colorectal cancer, Cheng et al³³ found that SIRT1 promotes EMT and metastasis by regulating Fra-1 expression. Recently, non-steroidal anti-inflammatory drugs were found to inhibit TGF- β 1-induced EMT via the downregulation of SIRT1.³⁴ However, SIRT1 has been documented to suppress EMT. In lung cancer, hypoxia promoted EMT and cancer metastasis by the repression of SIRT1 expression in a SUMOylation-dependent manner.³⁵ Consistent with this, in ovarian cancer, hypoxic stress induced EMT by the downregulation of SIRT1 transcription level.³⁶ In addition, SIRT1 has been shown to reduce EMT in breast cancer by

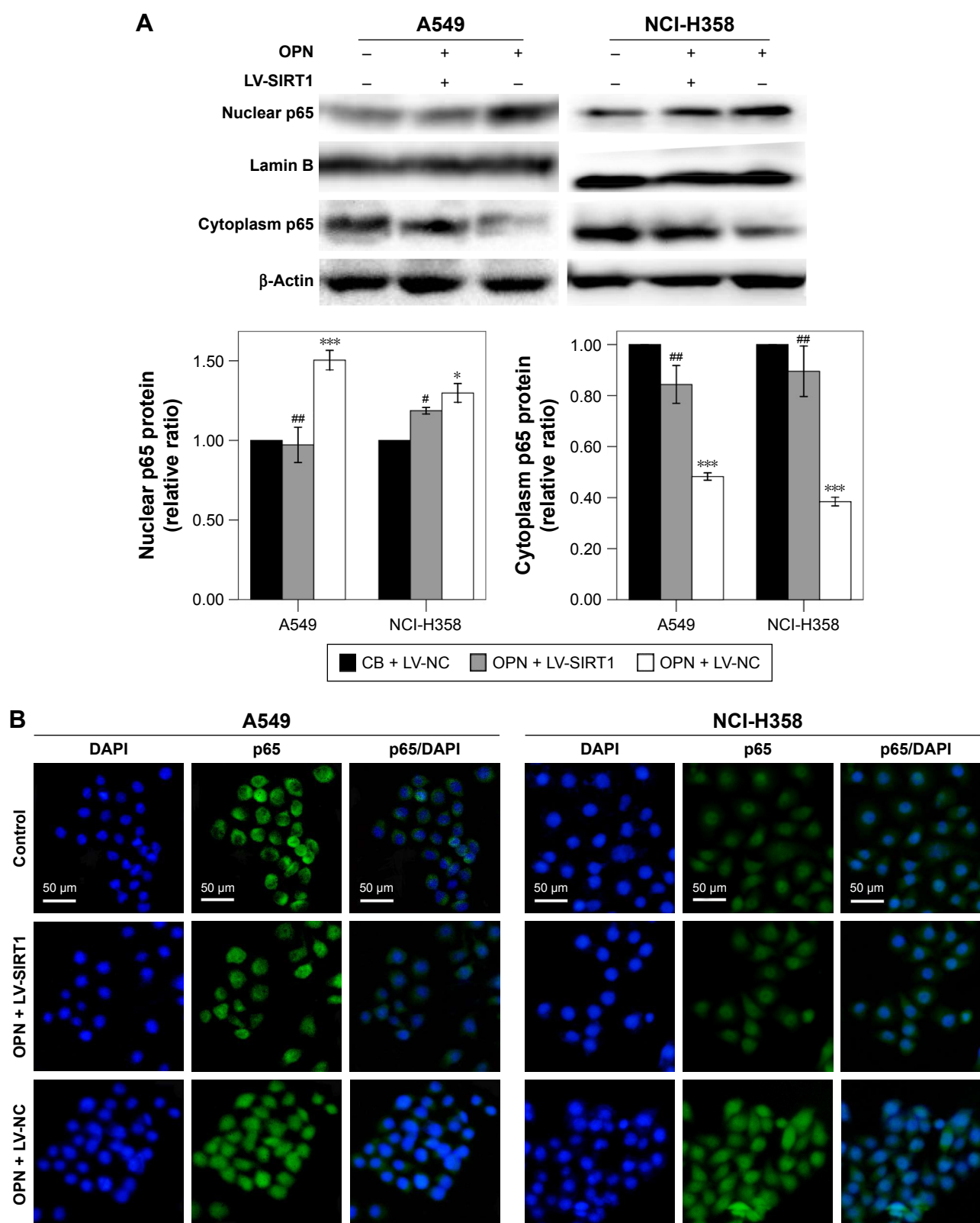


Figure 6 SIRT1 overexpression inhibits OPN-induced NF-κB activation.

Notes: A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with OPN (400 ng/mL) for 2 hours. **(A)** The levels of NF-κB p65 protein in cellular nucleus and cytoplasm were determined by Western blot analysis. Lamin B and β-Actin, respectively, indicate the nuclear and cytoplasmic fraction. The group treated with CB + LV-NC serving as control is presented as 1. Results presented are mean ± SD analyzed with Student's *t*-test from three independent experiments. **P* < 0.05, ****P* < 0.001 versus control. #*P* < 0.05, ###*P* < 0.01 versus OPN + LV-NC. **(B)** The locations of NF-κB p65 in the cells were examined by immunofluorescence. The group treated with CB + LV-NC served as control. The cells were immunostained with anti-NF-κB p65 antibody (green) and the nucleus was stained with DAPI. Scale bars represent 50 μm. Magnification ×100.

Abbreviations: SIRT1, Sirtuin 1; OPN, osteopontin; NF-κB, nuclear factor-κB; LV-SIRT1, lentiviral vectors overexpressing SIRT1; NC, negative control; CB, control buffer; SD, standard deviation.

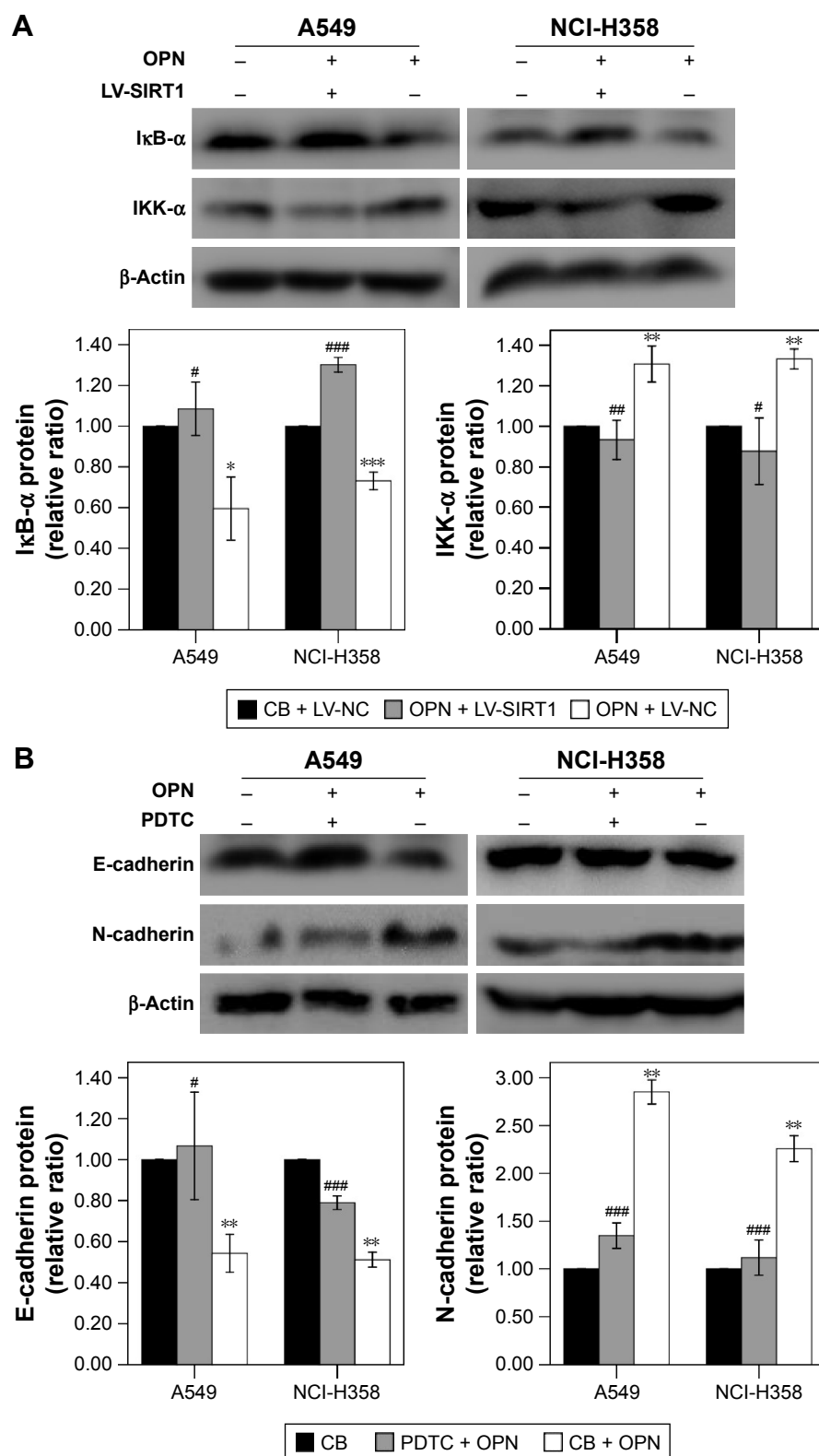


Figure 7 OPN promotes cell EMT via NF- κ B activation.

Notes: (A) A549 and NCI-H358 cells infected with LV-SIRT1 or LV-NC were treated with OPN (400 ng/mL) for 2 hours. The levels of I κ B- α and IKK- α were determined by Western blot analysis. β -Actin was used as an internal control. The group treated with CB + LV-NC serving as control is presented as 1. Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 versus CB + LV-NC. #*P*<0.05, ###*P*<0.01, ####*P*<0.001 versus OPN + LV-NC. (B) A549 and NCI-H358 cells were pretreated with NF- κ B inhibitor (PDTC, 10 μ M) for 30 minutes followed by treatment with OPN (400 ng/mL) for 24 hours. Western blot was used to detect E-cadherin and N-cadherin expression. β -Actin was used as an internal control. The group treated with CB serving as control is presented as 1. Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. ***P*<0.01 versus control. #*P*<0.05, ####*P*<0.001 versus CB + OPN.

Abbreviations: OPN, osteopontin; EMT, epithelial-mesenchymal transition; NF- κ B, nuclear factor- κ B; LV-SIRT1, lentiviral vectors overexpressing Sirtuin 1; NC, negative control; CB, control buffer; SD, standard deviation.

deacetylating Smad4 in the TGF- β pathway and repressing TGF- β signaling.²⁰ Therefore, it is likely that the effect of SIRT1 on EMT depends on the tumor types, specific downstream targets and tumor microenvironment. However, the underlying mechanisms of these contradictory data in different cell types and culture conditions would require further investigation.

OPN, as a secreted tumor regulatory protein in tumor microenvironment derived from both tumor cells and tumor associated stromal cells, mediates cell-matrix interactions and plays an important role in EMT initiation.³⁷ It is necessary to develop effective approaches to decrease OPN level or block the signaling cascades elicited by OPN. The SIRT1 activator, resveratrol, takes part in anti-nephrolithic activity mediated partially by the inhibition of OPN.³⁸ However, the relationship between OPN and SIRT1 in tumor is still unclear. Recent studies have showed that SIRT1 expression was regulated by various cytokines, such as TGF- β , TNF- α and IL-1 β .^{30,39,40} This is the first study to demonstrate that SIRT1 expression was decreased in NSCLC cells treated with OPN. Since NF- κ B is a direct target of SIRT1,¹⁶ the effect of SIRT1 overexpression on NF- κ B signaling pathway activated by OPN was explored. It was discovered that SIRT1 overexpression inhibits OPN-induced NF- κ B activation. Previous studies have demonstrated that SIRT1 suppresses tumor cell proliferation and reduces chemoresistance by inhibiting NF- κ B signaling.^{21,22} In our study, the results exhibited that SIRT1 overexpression could inhibit OPN-induced NSCLC cell EMT by the inhibition of NF- κ B activation.

Conclusion

In conclusion, SIRT1 expression was inhibited and NF- κ B acetylation was promoted by OPN treatment. Our study suggests that SIRT1 is a potential tumor suppressor that inhibits OPN-induced NF- κ B activation by deacetylation of NF- κ B p65 to protect NSCLC cells from OPN-induced EMT. Therefore, SIRT1 may be a novel therapeutic target for treating OPN-induced NSCLC progression.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

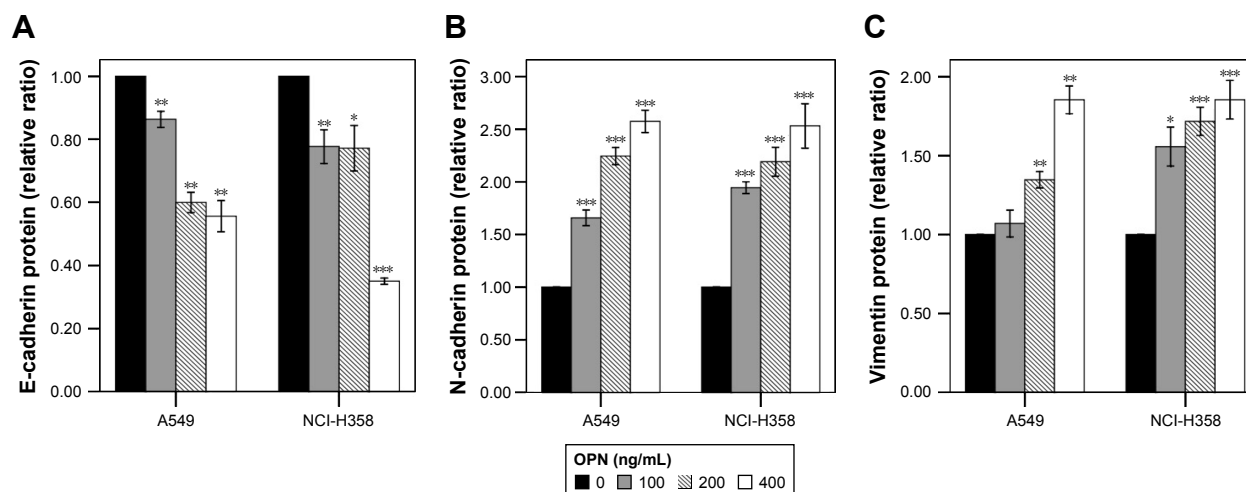


Figure S1 OPN promotes NSCLC cell EMT.

Notes: (A–C) The protein expression of target genes in control group is presented as 1. Results presented are mean \pm SD analyzed with Student's *t*-test from three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control.

Abbreviations: OPN, osteopontin; NSCLC, non-small cell lung cancer; EMT, epithelial-mesenchymal transition; SD, standard deviation.

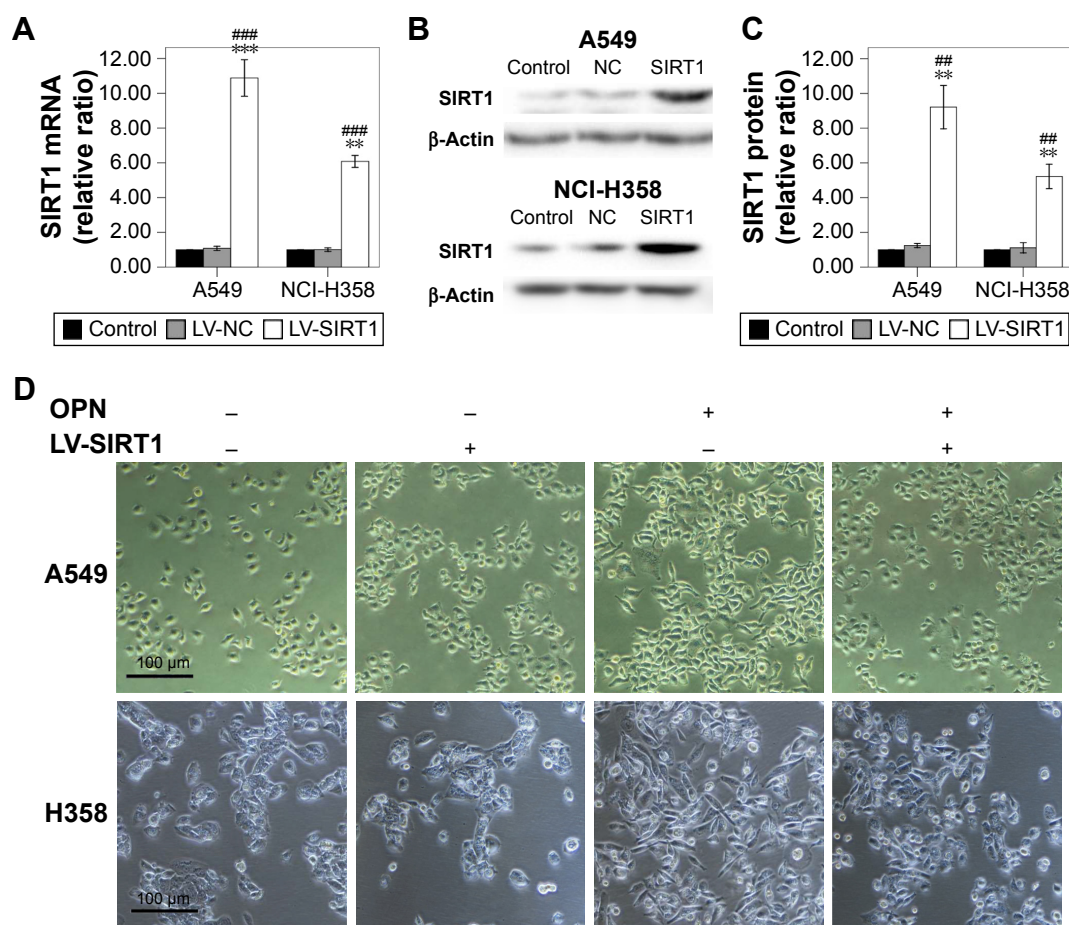


Figure S2 Overexpression of SIRT1 inhibits OPN-induced changes of cellular morphology.

Notes: (A–C) A549 and NCI-H358 cells were infected with LV-SIRT1 or LV-NC. The effect of the infection was evaluated by real-time qPCR and Western blot analysis at 96 hours after infection. The cells without lentiviral infection served as control. The mRNA and protein expression of SIRT1 in control group is presented as 1. ***P* < 0.01, ****P* < 0.001 versus control. ###*P* < 0.01, ####*P* < 0.001 versus LV-NC. (D) Cells infected with LV-SIRT1 or LV-NC were treated with OPN (400 ng/mL) for 72 hours; the cells were photographed. Representative images ($\times 100$) are presented. Scale bars represent 100 μ m.

Abbreviations: SIRT1, Sirtuin 1; OPN, osteopontin; LV-SIRT1, lentiviral vectors overexpressing SIRT1; NC, negative control; qPCR, quantitative polymerase chain reaction.

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