

Sirtuin 1 attenuates nasal polypogenesis by suppressing epithelial-to-mesenchymal transition

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Background: Nasal polyps (NPs) imply a refractory clinical course in patients with chronic rhinosinusitis (CRS). Previously, we showed that hypoxia-inducible factor (HIF) 1 could mediate nasal polypogenesis through epithelial-to-mesenchymal transition (EMT). Sirtuin 1 (SIRT1), a histone deacetylase, reportedly suppresses the transcriptional activity of HIF-1. Thus we hypothesized that SIRT1 attenuates nasal polyposis by inhibiting HIF-1-induced EMT.

Objective: We sought to determine the role of SIRT1 in patients with nasal polyposis.

Methods: The effects of SIRT1 on nasal polypogenesis were investigated in previously developed murine models. Immunohistochemistry, immunoblotting, and immunoprecipitation were done to evaluate SIRT1, EMT, and hypoxic markers in human nasal epithelial cells or sinonasal tissues from the mice and the patients with CRS with or without NPs.

Results: SIRT1 transgenic mice had significantly fewer mucosal lesions with epithelial disruption and fewer NPs than wild-type (WT) mice. In addition, resveratrol (a SIRT1 activator) treatment suppressed nasal polypogenesis in WT mice; however, sirtinol (a SIRT1 inhibitor) administration increased the polyp burden in SIRT1 transgenic mice. In sinonasal specimens from patients with CRS, SIRT1 was downregulated in the mucosa from patients with polyps compared with levels seen in patients without polyps. SIRT1 overexpression or activation reversed

hypoxia-induced EMT in human nasal epithelial cells. The intranasal transfection of a small hairpin SIRT1 lentiviral vector induced more nasal polypoid lesions in SIRT1 transgenic mice. Finally, mucosal extracts from patients with CRS without NPs increased SIRT1 expression in nasal epithelial cells, whereas those from patients with CRS with NPs did not. **Conclusion:** SIRT1 suppressed NP formation, possibly because of inhibition of HIF-1-induced EMT. Thus nasal epithelium SIRT1 might be a therapeutic target for NPs. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Nasal polyposis, epithelial-to-mesenchymal transition, sirtuin 1, hypoxia-inducible factor 1, animal model

Chronic rhinosinusitis (CRS) is characterized by chronic inflammation of the nasal and paranasal sinus mucosa and related to mucosal alterations ranging from inflammatory thickening to nasal polyp (NP) formation.^{1,2} CRS is frequently divided into 2 groups based on the absence or presence of NPs²: chronic rhinosinusitis with nasal polyps (CRSwNP)¹ and chronic rhinosinusitis without nasal polyps (CRSsNP). Histomorphological features of NPs include the subepithelial accumulation of inflammatory cells with a prominent rate of eosinophils, frequent epithelial damage, and a thickened basement membrane.³ The formation of NPs, which is associated with T_H2-skewed inflammation, particularly in Western countries, implies a greater burden of illness with refractory clinical features.^{4,5} However, the mechanisms underlying mucosal remodeling and polyp formation in patients with CRSwNP are unclear.

A hypoxic environment affects mucosal inflammation and epithelial remodeling and promotes the development of NPs.⁶⁻⁸ In particular, we reported that hypoxia-inducible factor (HIF) 1 mediated nasal polypogenesis by inducing epithelial-to-mesenchymal transition (EMT) and suggested that HIF-1 α be viewed as a therapeutic target for nasal polyposis.⁸ In addition, hypoxia-induced vascular endothelial growth factor (VEGF) production contributed to mucosal edema in NPs; HIF-1 α inhibitors effectively suppressed VEGF secretion.⁷ It was also suggested that HIF-1 α regulated VEGF to perpetuate the hyperplastic epithelial growth observed in patients with CRSwNP.⁹ Although several HIF-1 inhibitors were introduced for the treatment of hypoxia-related diseases, most were developed as anticancer drugs and thus were cytotoxic.¹⁰ Given that CRSwNP is not life-threatening, application of these potent HIF-1-modulating drugs could lead to safety issues.

Sirtuin (Sir2), which is reportedly involved in gene silencing and lifespan extension, was originally identified in yeast and *Drosophila* species.¹¹ Sirtuin 1 (SIRT1) reportedly fine-tuned cellular responses to hypoxia by deacetylating HIF-1 α and HIF-2 α .¹² Among the seven known mammalian Sir2 homologs (Sirt1-7), SIRT1 is considered the prototypical sirtuin because it

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Abbreviations used

CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
EMT:	Epithelial-to-mesenchymal transition
ENP:	Eosinophilic nasal polyp
HIF:	Hypoxia-inducible factor
hNEC:	Human nasal epithelial cell
HPF:	High-power field
NENP:	Noneosinophilic nasal polyp
NF-κB:	Nuclear factor κB
NP:	Nasal polyp
OVA:	Ovalbumin
PKD1:	Pyruvate dehydrogenase lipoamide kinase isozyme 1
SEB:	<i>Staphylococcus aureus</i> enterotoxin B
SIRT1:	Sirtuin 1
α-SMA:	α Smooth muscle actin
UP:	Uncinate process
VEGF:	Vascular endothelial growth factor
WT:	Wild-type

is most homologous to Sir2.¹³ SIRT1 functions to remove the acetyl group from acetylated lysine residues in both histones and nonhistone proteins. As a consequence of SIRT1-mediated deacetylation, HIF-1α transcriptional activity is suppressed because of p300 dissociation from the C-terminal transactivation domain of HIF-1α.¹²⁻¹⁴ In addition, diverse drugs, chemicals, or both activate or inhibit the activity of Sirt1 and are readily available.¹⁵ Thus we speculated that Sirt1 might be a therapeutic target for the treatment of nasal polyposis by inhibiting HIF-1-induced tissue remodeling. Some of the results of this study were reported previously in abstract form.¹⁶

METHODS**Human subjects**

All subjects studied were enrolled after providing written informed consent under the internal review board of an SNUH Boramae Medical Center–approved protocol (no. 06-2012-109). The diagnosis of CRS with or without polyps was based on historical, endoscopic, and radiographic criteria.¹⁷ The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography of the paranasal sinuses. NPs were confirmed by using nasal endoscopic findings and signs of persistent bilateral and diffuse paranasal sinus mucosal thickening in computed tomographic scans. Endoscopic sinus surgery was performed when the patient's symptoms and radiographic findings did not resolve at least 6 weeks after patients were treated with antibiotics, topical corticosteroids, decongestants, and/or mucolytic agents. Antibiotics and topical steroids were discontinued 2 weeks before surgery. No patients took oral corticosteroids at the time of their surgery. Patients with acute infection were excluded in this study. The patients with a deviated nasal septum were considered the control group. Demographic findings of patients are summarized in Table I. Mucosal extracts from patients with deviated nasal septum only, CRSsNP, and CRSwNP were isolated and used for *in vitro* studies. NPs were classified as eosinophilic when the percentage of eosinophils among all immune cells in tissues exceeded 10%, which was chosen as the cutoff in the previous report.¹⁸ Detailed procedures are described in the **Methods** section in this article's Online Repository at www.jacionline.org.

Culture of epithelial cell lines

Normal human nasal epithelial cells (hNECs) were purchased from PromoCell (Heidelberg, Germany). The RPMI2650 cell line, which originated

from upper airway epithelial cells, was obtained from the Korean Cell Line Bank (Seoul, Korea). hNECs were cultured in airway epithelial cell growth medium from PromoCell. RPMI 2650 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, respectively. hNECs were used for analysis within 5 passages after purchase. All cells were grown in 5% CO₂/20% O₂ (normoxic) or 5% CO₂/1% O₂ (hypoxic).

Immunohistochemistry

SIRT1, HIF-1α, and EMT markers were immunostained in paraffin sections (4 μm) of sinonasal tissues. Detailed immunohistochemistry procedures are described in the **Methods** section in this article's Online Repository.

ELISA for mouse ovalbumin-specific IgE

Serum levels of ovalbumin (OVA)–specific IgE in mice were quantified by using the LEGEND MAX ELISA kit provided by BioLegend (San Diego, Calif). Each assay was performed in triplicate.

Immunoblotting and immunoprecipitation

Proteins were separated on 8–12% SDS/polyacrylamide gels, transferred to Immobilon-P membranes, and sequentially incubated with primary and secondary antibodies. Detailed information is described in the **Methods** section in this article's Online Repository. For immunoprecipitation, cell lysates were incubated sequentially with 1 μg of antibodies and 20 μL of protein A/G sepharose beads for 4 hours at 4°C (Santa Cruz Biotechnology, Dallas, Tex). Then immunoprecipitates were eluted with a sample buffer (1% SDS, 100 mmol/L dithiothreitol, and 50 mmol/L Tris, pH 7.5) and subjected to immunoblotting. Densitometric analysis was performed on the scanned images of blots by using the ImageJ software program (National Institutes of Health Image processing analysis, <http://rsb.info.nih.gov/ij/>).

Plasmid DNA and small hairpin RNA lentiviral vector transfection

Cells at 60% confluence were transfected with Myc-His-SIRT1 plasmid by using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, Calif) or small interfering RNAs by using RNAi-MAX Reagent (Invitrogen). The Myc-His-SIRT1 plasmid was kindly provided by Dr Junjie Chen (University of Texas). For gene silencing, the pLKO.1-puro vector was purchased from Sigma-Aldrich (St Louis, Mo), and oligonucleotides targeting the green fluorescent protein (control small hairpin RNA) or SIRT1 were inserted into the vector by using *AgeI* and *EcoRI* restriction enzymes. The construct was confirmed by means of sequencing. The viral vector was cotransfected with pMD2-VSVG, pRSV-RRE, and pMDLg/pRRE helper DNA into HEK293T cells, and the viral supernatant was collected. Detailed protocols are described in the **Methods** section in this article's Online Repository.

A murine NP model

C57BL/6J mice (5 weeks old) were purchased from Central Laboratory Animal (Seoul, Korea) and kept in specific pathogen-free rooms. SIRT1 transgenic mice were established with Myc-His tagged SIRT1 plasmid with a CMV promoter. Detailed information is described in Fig E1 in this article's Online Repository at www.jacionline.org. All experimental protocols complied with the Guidelines of the National Institute of Health and the Declaration of Helsinki and were approved by the Committee on the Use and Care of Animals. A murine NP model was generated from an allergic rhinosinusitis model in mice with minor modification, as described previously.¹⁹ Briefly, mice were immunized with an intraperitoneal injection of 25 μg of OVA (Sigma) in 2 mg of aluminum hydroxide gel on days 0 and 5, followed by a daily intranasal instillation from days 12 to 19 with 6% OVA diluted in PBS. Thereafter, prolonged continuous inflammation was maintained in the experimental mice by means of subsequent nasal exposure of mice to 6% OVA 3 times a week for 12 consecutive weeks. In addition

TABLE I. Subjects' characteristics

	Control subjects	Patients with CRSsNP	Patients with CRSwNP	
Total no. of subjects	11	15	10	21
Tissue used	UP	UP	UP	NP
Age (y), mean (SD)	49 (20)	45 (15)	49 (14)	53 (13)
Atopy, no. (%)	1 (9)	2 (13)	1 (10)	3 (14)
Asthma, no.	0	1	0	2
Aspirin sensitivity, no.	0	0	0	0
Lund-Mackay CT score, mean (SD)	0 (0)	9.3 (5.5)	12.7 (5.3)	13.1 (5.2)
Blood eosinophils (cells/mm ³)	67.9 (33.1)	139.7 (95.9)	163.6 (132.9)	150.7 (108.2)
Methodology used, no.				
Immunohistochemistry	8 (6 male)	12 (6 male)	10 (7 male)	21 (17 male)
Tissue extracts	3 (3 male)	3 (1 male)	—	8 (5 male)

to 6% OVA, all groups of mice were challenged 3 times a week with 10 ng of *Staphylococcus aureus* enterotoxin B (SEB; List Biological laboratories, Campbell, Calif) from the 8th through the 14th week after OVA instillation. The control groups were designated as follows: instillation with PBS only (negative control) and instillation with 6% OVA plus 10 ng of SEB plus vehicle (NP control). The experimental groups were designated as follows: instillation with 6% OVA plus SEB plus chemicals (1 μ g of resveratrol or sirtinol) or lentiviral vectors (small hairpin [sh]-tGFP or sh-SIRT1; see corresponding figures for detailed schedule). The sinonasal specimens were collected and processed, as described previously.¹⁹ Detailed procedures are described in the **Methods** section in this article's Online Repository. Polyp formation and epithelial disruption were microscopically examined from 3 coronal sections. The criteria for NPs included (1) more elevated lesion than surrounding mucosal folds, (2) eosinophilic infiltration, and (3) microcavities.

Statistical analysis

Statistical analyses used in this study include the Mann-Whitney *U* test with the 2-tailed test for unpaired comparisons, the Wilcoxon signed rank test, the Pearson correlation test, and linear regression, which were performed with IBM SPSS 21 software (SPSS, Chicago, Ill) and GraphPad Prism software 6.0 (GraphPad Software, La Jolla, Calif). Pearson correlations were used to determine variable relationships. If not normally distributed, the Spearman correlation coefficient was selected. All statistical tests were 2-sided, and significance was considered when the *P* value was less than .05.

RESULTS

Reduced polyp burden in SIRT1 transgenic mice

First, we investigated the effect of Sirt1 overexpression on NP formation using a previously established murine NP model.¹⁹ As shown in **Fig 1, A**, C57BL/6 wild-type (WT) and SIRT1 transgenic mice were treated with OVA and SEB to induce NP formation (see the **Methods** section for a detailed explanation). Interestingly, OVA and SEB treatment did not induce any marked mucosal changes in transgenic mice but led to prominent polypoid

lesions in WT mice (**Fig 1, B**). Mucosal polyp numbers in the nasal cavities and epithelial disruptions were significantly reduced in SIRT1 transgenic mice compared with WT mice (**Fig 1, C and D**).

Reciprocal expression of SIRT1 and HIF-1 α in tissue from patients with CRS

We previously found that HIF-1 α induced E-cadherin loss in NP epithelial cells.⁸ To verify the relationship between SIRT1 and HIF-1 α , both proteins were analyzed by using immunohistochemistry. In uncinat process (UP) specimens from patients with deviated nasal septa (control subjects) or CRSsNP, marked E-cadherin-positive staining was observed in the mucosal epithelia, whereas HIF-1 α was weakly detected. However, E-cadherin expression was downregulated and nuclear HIF-1 α expression was upregulated in both UP and NP tissues harvested from patients with CRSwNP (**Fig 2, A, C, and D**). More SIRT1-positive nuclei were detected in UP from patients with CRSsNP than in UP from control subjects; SIRT1-positive nuclei were markedly reduced in CRSwNP NPs (**Fig 2, A and B**). Furthermore, nuclear SIRT1 levels were positively correlated with E-cadherin expression (**Fig 2, E**). These findings imply that SIRT1 and HIF-1 α were reciprocally expressed and that Sirt1 could contribute to the repression of HIF-1 α -mediated E-cadherin loss.

Effects of an SIRT1 activator or inhibitor on polyp formation in a murine model

Next, we evaluated the effects of SIRT1 on polypogenesis in WT and SIRT1 transgenic mice through intranasal treatment with a SIRT1 activator (resveratrol) and inhibitor (sirtinol), respectively. All mice were treated with OVA and SEB to induce NPs according to a previously described protocol. Additionally, 1 μ g of each drug was instilled 3 times per week from day 47 until the end of the experiment, as shown in **Fig 3, A**. Resveratrol instillation in WT mice significantly suppressed polyp formation (**Fig 3, B**) and also reduced the number of mucosal goblet cells (**Fig 3, C**). On the contrary, sirtinol intranasal treatment in SIRT1 transgenic mice increased polyp formation (**Fig 3, B**) and the number of goblet cells (**Fig 3, C**). Total IgE levels were decreased after resveratrol treatment in WT mice and increased by sirtinol administration in SIRT1 transgenic mice (**Fig 3, D**). Thus polypoid changes induced by OVA and SEB in mice were affected by intranasal treatment with SIRT1 modulators.

SIRT1 attenuates HIF-1 α activity through deacetylation in nasal epithelial cells

We then investigated the molecular mechanism underlying the suppressive effect of Sirt1 on polypogenesis *in vitro*. When cultured under hypoxic conditions (1% O₂), human nasal epithelial RPMI 2650 cells underwent EMT, as evidenced by a decrease in epithelial markers (E-cadherin and β -catenin) and an increase in mesenchymal markers (α smooth muscle actin [α -SMA] and vimentin) and known EMT-inducing factors (TWIST, ZEB, and SNAIL; **Fig 4, A**). SIRT1 overexpression reversed this hypoxia-induced EMT in RPMI 2650 cells (**Fig 4, A**). Similarly, resveratrol, an SIRT1 activator, inhibited hypoxia-induced EMT in both RPMI 2650 cells and hNECs (**Fig 4, B**). In addition, hNEC cellular morphology was restored after resveratrol treatment (**Fig 4, C**). We reported previously

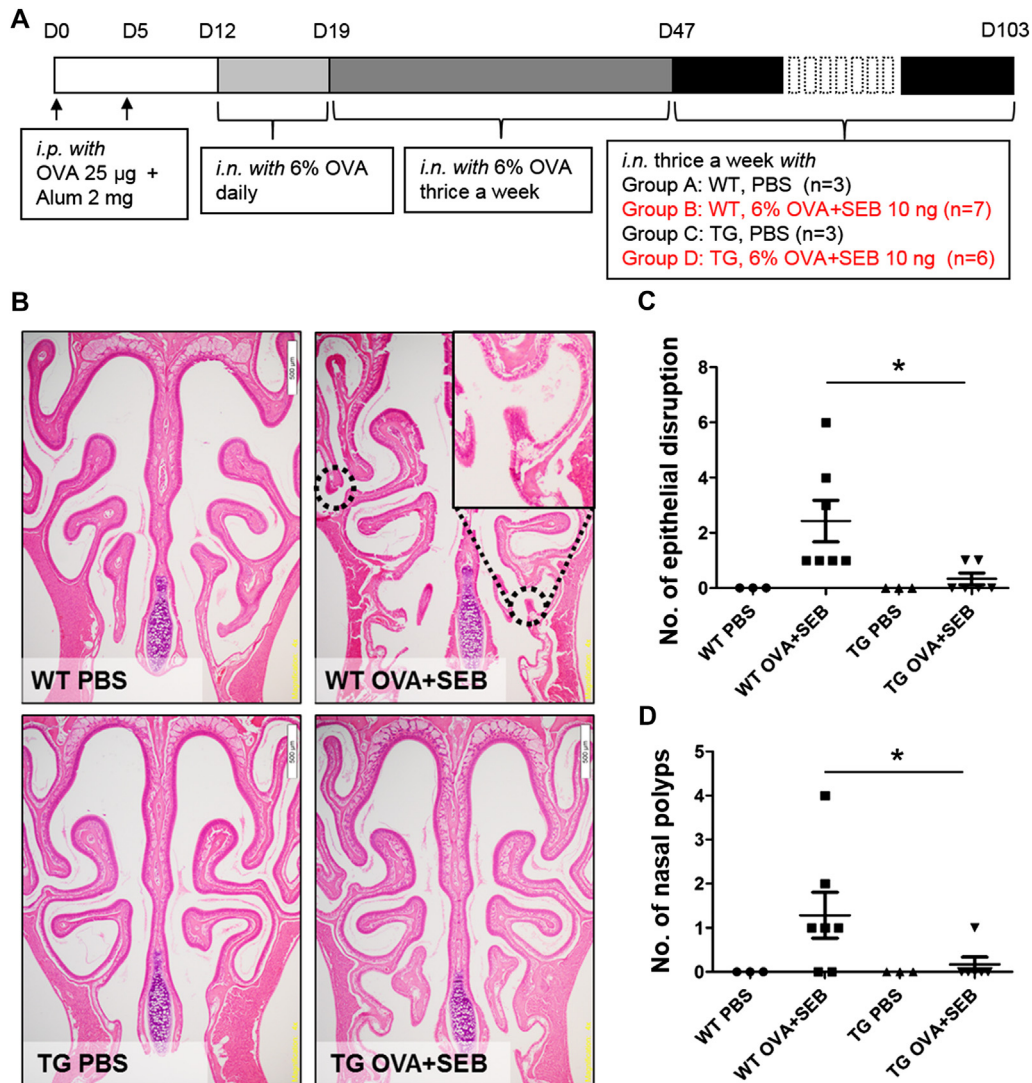


FIG 1. Polyp burden is reduced in SIRT1 transgenic (TG) mice. **A**, Protocol for the murine NP model. WT and SIRT1 transgenic mice were treated with OVA and SEB. *i.p.*, Intraperitoneal; *i.n.*, intranasal. **B**, Photographs of representative sinonasal spaces and polypoid lesions stained with hematoxylin and eosin. The criteria for NPs are described in the [Methods](#) section, and dotted circles indicate polypoid lesions. **C** and **D**, Numbers of epithelial disruptions and polyps were expressed as the total number per coronal section. * $P < .05$, Mann-Whitney U test.

that HIF-1 α could mediate hypoxic EMT in hNECs.⁸ Interestingly, HIF-1 α and its well-known downstream target, pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1), were suppressed by SIRT1 upregulation (Fig 4, A and B). Next we questioned whether HIF-1 α could be inhibited by SIRT1 activation in hypoxia-independent conditions. When HIF-1 α was upregulated by the direct transfection or IFN- γ treatment (HIF-1 α induction by IFN- γ in upper airway cells was found in our recent study; see Fig E2 in this article's Online Repository at www.jacionline.org), resveratrol could downregulate HIF-1 α and EMT in RPMI 2650 cells (see Fig E3 in this article's Online Repository at www.jacionline.org). Considering that SIRT1 inhibited HIF-1 α through its deacetylation,¹² we examined the influence of SIRT1 activity on HIF-1 acetylation in RPMI 2650 cells. Using a previously developed antibody against K674-acetylated HIF-1 α ,¹⁴ we confirmed that HIF-1 α

acetylation was diminished by activating SIRT1 both in normoxic and hypoxic conditions (Fig 4, D and E, and see Fig E4 in this article's Online Repository at www.jacionline.org). These data suggested that SIRT1 could inhibit HIF-1 α -treated EMT in hNECs by suppressing HIF-1 α expression and transcriptional activity.

Tissue-specific SIRT1 knockdown restores polyp formation in SIRT1 transgenic mice

To avoid the effects of whole-body SIRT1 expression in transgenic mice, we manipulated SIRT1 in a tissue-specific manner by using lentiviral vectors in our murine polyp model (Fig 5, A). As expected, SIRT1 transgenic mice bore fewer inflammatory mucosal changes than WT mice, but the intranasal administration of sh-SIRT1 lentiviral vectors in SIRT1 transgenic

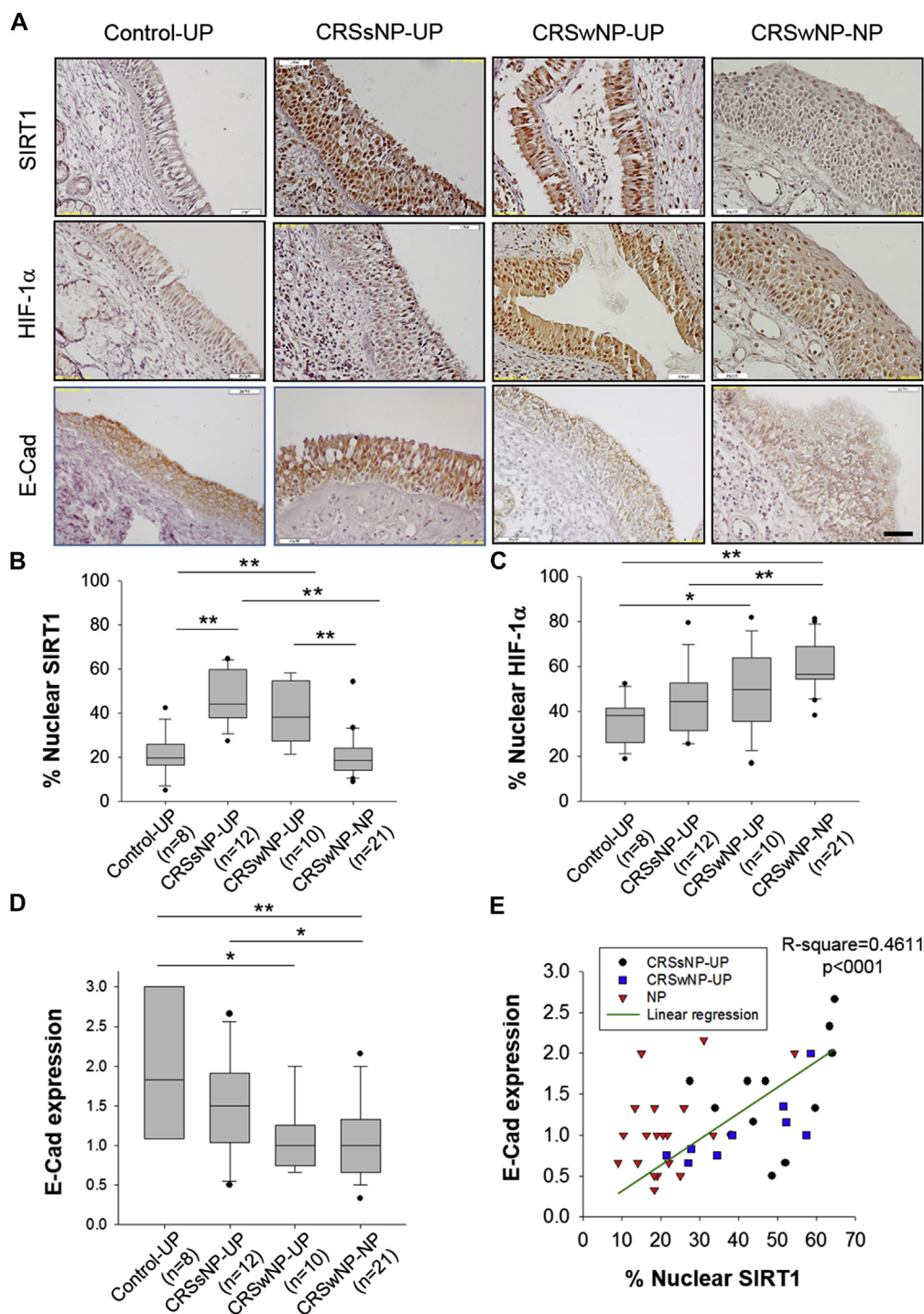


FIG 2. Reciprocal expression of SIRT1 and HIF-1α in patients with CRSwNP or CRSsNP. **A**, Control UP mucosa from patients without nasal disease, UPs from patients with CRSsNP, and both UP and NP tissues from patients with CRSwNP were immunostained with SIRT1, HIF-1α, and E-cadherin (*E-Cad*) antibodies. Scale bars = 100 μm. **B-D**, Comparison of nuclear HIF-1α, SIRT1, and E-cadherin expression in control UPs, UPs from patients with CRSsNP, UPs from patients with CRSwNP, and NPs. Detailed criteria for scoring are described in the [Methods](#) section in this article's Online Repository. **P* < .05 and ***P* < .01, Mann-Whitney *U* test. **E**, Relationship between nuclear SIRT1 and E-cadherin expression. The Pearson correlation test was used, and *R*² represents the coefficient of determination.

mice restored distinct mucosal inflammation with multiple polypoid lesions (Fig 5, B). The change in SIRT1 expression was confirmed by performing immunohistochemistry against

SIRT1 (Fig 5, C). The number of epithelial disruptions and NPs was also reversed by the nasal mucosa-specific knockdown of SIRT1 in transgenic mice (Fig 5, D and E).

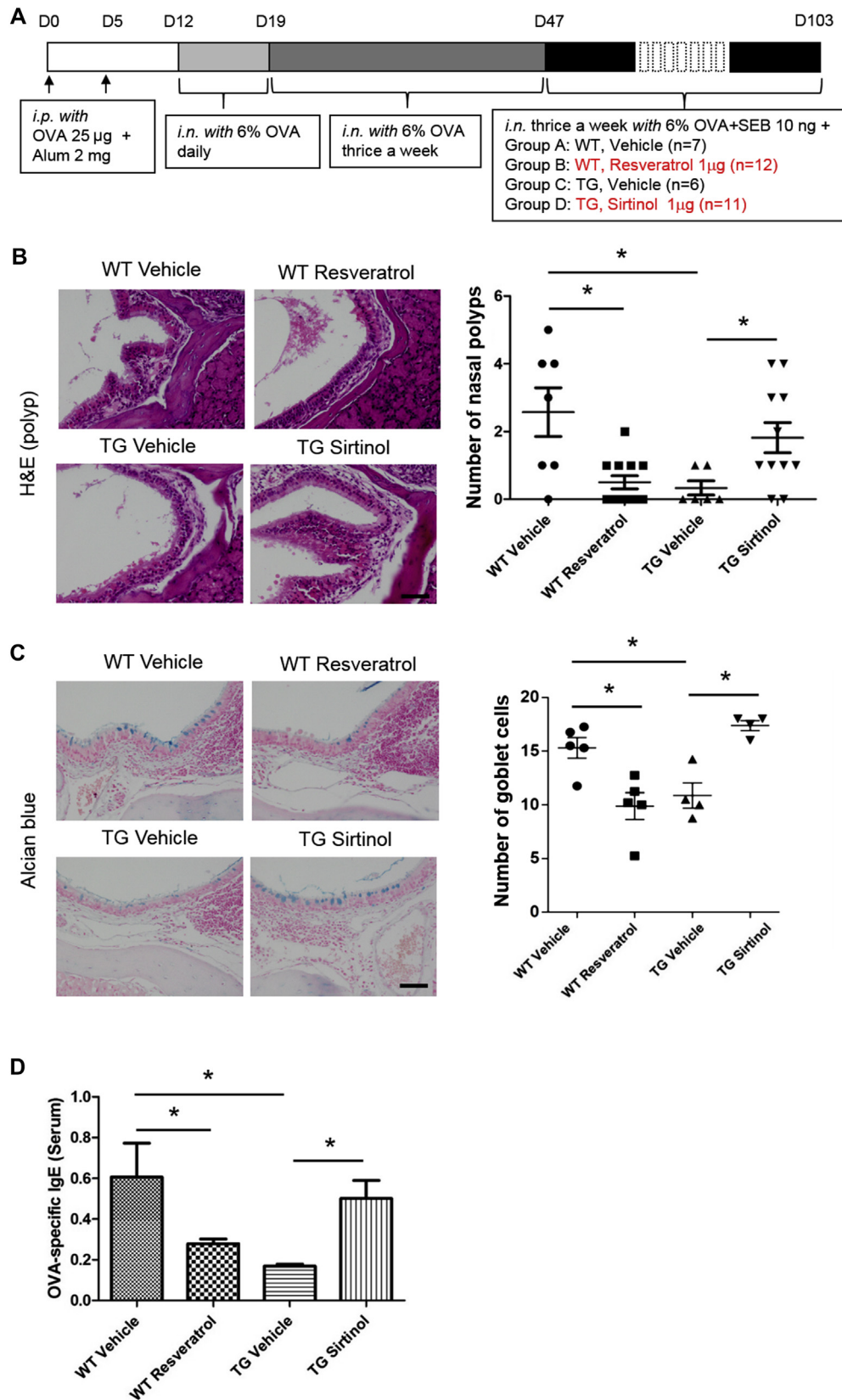


FIG 3. Effect of an SIRT1 activator or inhibitor on polyp formation in mice. **A**, Protocol for the murine NP model. *i.p.*, Intraperitoneal; *i.n.*, intranasal. **B**, Representative photographs and numbers of polypoid lesions stained with hematoxylin and eosin. NP criteria are described in the [Methods](#) section. **C** and **D**, Representative photographs and numbers of goblet cells stained with Alcian blue. Fig 3, **D**, shows OVA-specific IgE levels in mouse serum. * $P < .05$, Mann-Whitney U test. Scale bar = 50 μ m.

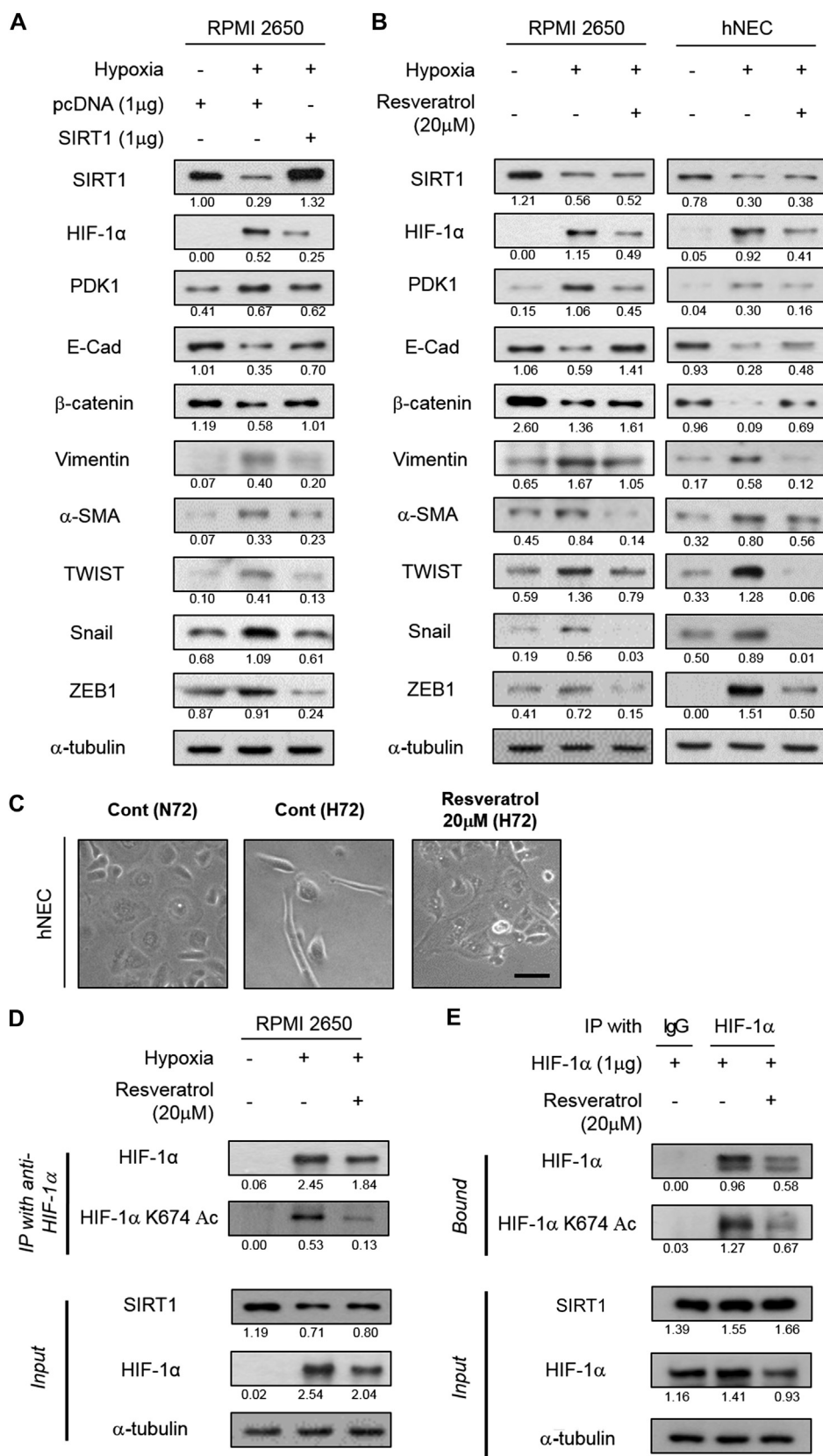


FIG 4. SIRT1 suppresses hypoxia-induced EMT by deacetylating HIF-1 α . **A**, RPMI 2650 cells were transfected with 1 μ g of pcDNA or SIRT1 plasmid and incubated under normoxic or hypoxic conditions for 72 hours. EMT markers were immunoblotted. The intensity values of bands were normalized by α -tubulin expression. **B** and **C**, RPMI 2650 and A549 cells were treated with resveratrol or vehicle and incubated under normoxic or hypoxic conditions for 72 hours. EMT markers were immunoblotted, and phase-contrast images of hNECs were acquired. Scale bar = 100 μ m. **D** and **E**, RPMI 2650 cells were treated with resveratrol or vehicle in the HIF-1 α -overexpressed or hypoxic conditions (24 hours). Whole-cell lysates and immunoprecipitated proteins were immunoblotted with the indicated antibodies. H, Hypoxic; N, normoxic.

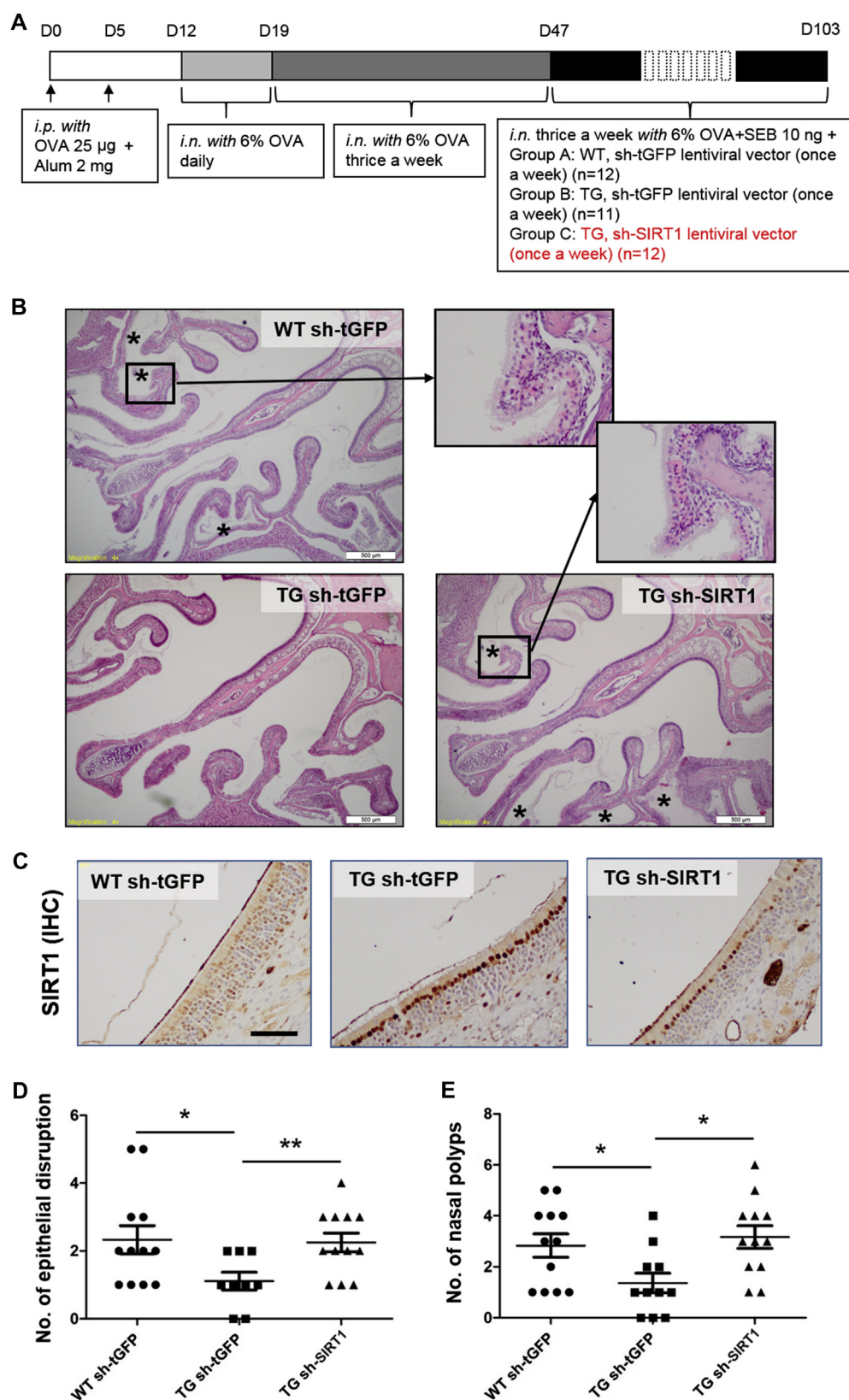


FIG 5. Tissue-specific knockdown of SIRT1 restores polyp formation in SIRT1 transgenic (TG) mice. **A**, Protocol for the murine NP model. WT and SIRT1 transgenic mice were treated with OVA, SEB, and sh-tGFP or sh-SIRT1 lentiviral vectors. *i.p.*, Intraperitoneal; *i.n.*, intranasal. **B**, Photographs of representative polypoid lesions stained with hematoxylin and eosin in the indicated groups. **C**, Photographs of representative immunohistochemical staining against SIRT1. **D** and **E**, Numbers of epithelial disruptions and polypoid lesions were compared. * $P < .05$ and ** $P < .01$, Mann-Whitney U test.

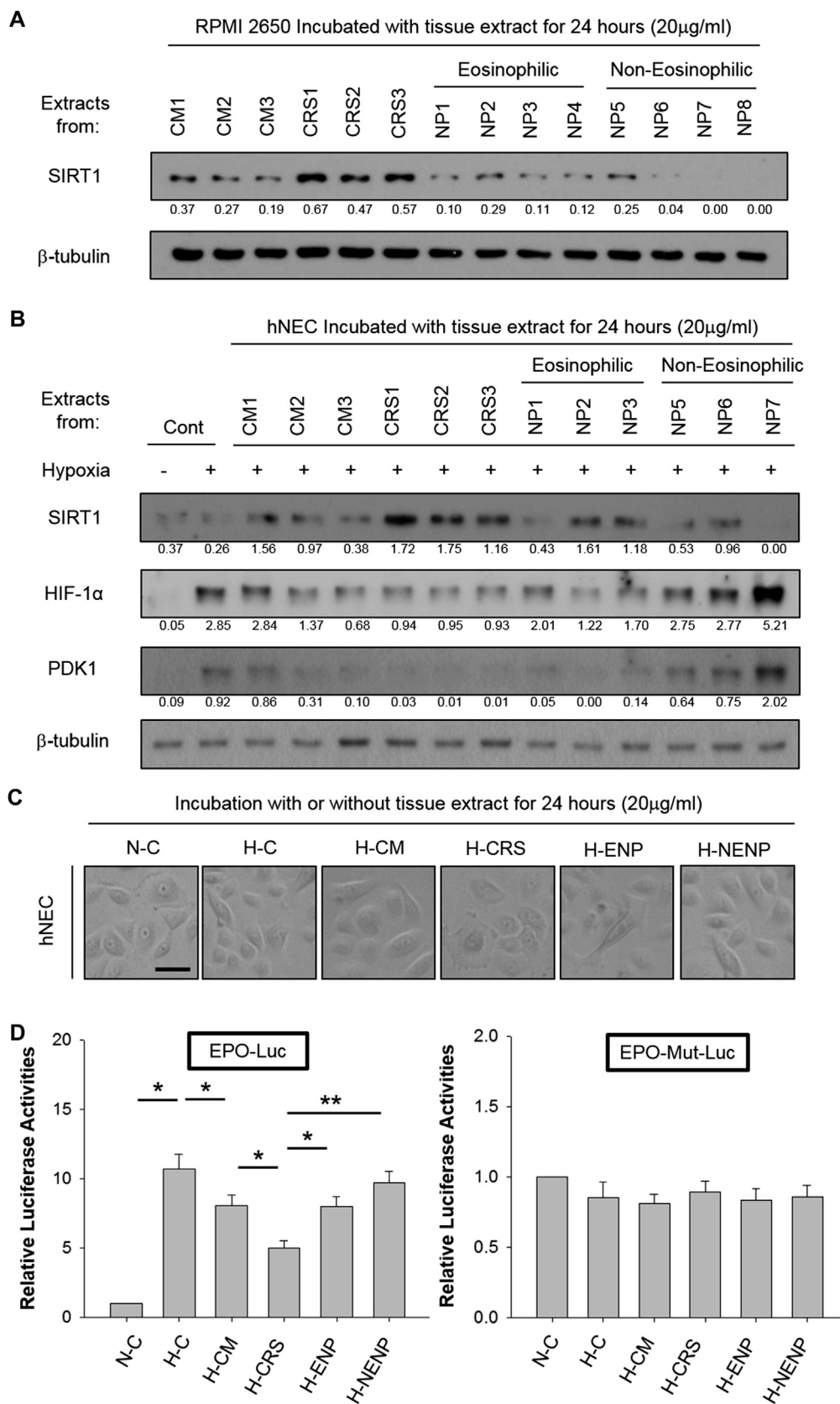


FIG 6. Increased expression of SIRT1 in nasal epithelial cells treated with mucosal extracts from patients with CRSsNP but not those with CRSwNP. **A**, SIRT1 expression in RPMI 2650 cells treated with mucosal extracts from control UPs without nasal pathology, UPs of patients with CRS, or NP tissues from patients with CRSwNP. The intensity values of bands were normalized by β -tubulin expression. **B** and **C**, hNECs were incubated under normoxic or hypoxic conditions with mucosal extracts from UPs, patients with CRS, or NPs. hNEC phase-contrast images were acquired. Scale bar = 100 μ m. **D**, Epo-luciferase and Epo-mutant luciferase activities with mucosal extracts (20 μ g/mL) from UPs, patients with CRS, or NPs as above. * P < .05 and ** P < .01, Mann-Whitney U test. H, Hypoxic; N, normoxic.

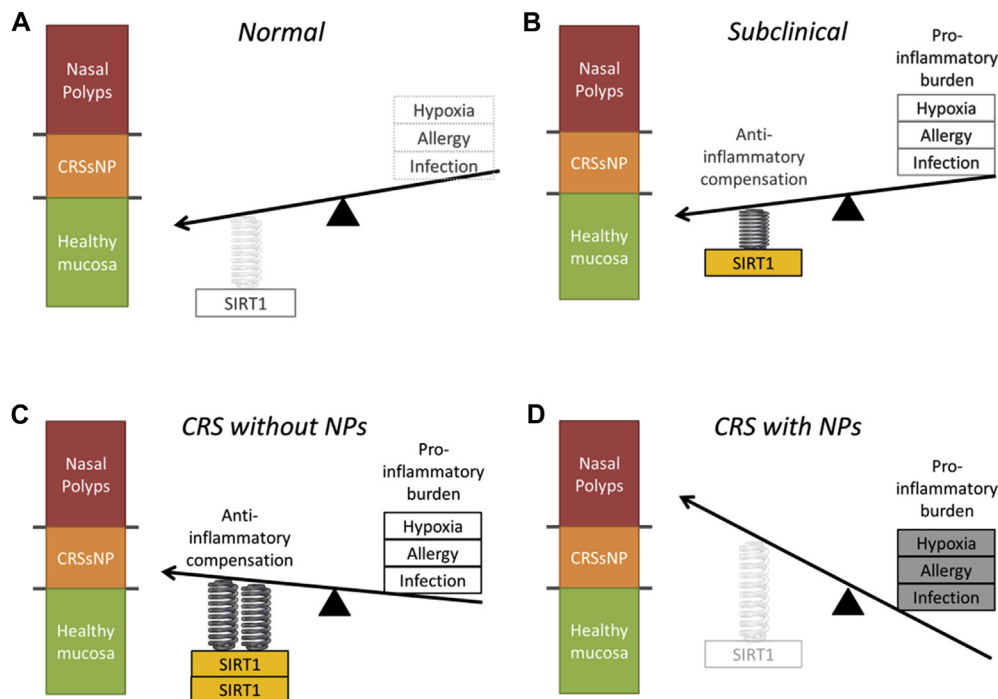


FIG 7. Schematic illustration of the role of SIRT1 in patients with CRSwNP. In healthy mucosa (**A**), there is less proinflammatory burden, so SIRT1 expression is very weak. With the subclinical inflammation in sino-nasal mucosa (**B**), SIRT1 could be expressed and compensate the inflammation. Under the persistent or severe inflammation as like CRS (**C**), SIRT1 could be induced more but may not fully compensate the proinflammatory drives. Thus, the loss of SIRT1 could lead the substantial inflammatory surge and finally nasal polyp formation (**D**).

SIRT1 expression is enhanced by mucosal extracts from patients with CRS but not by extracts from NPs

Finally, we examined the effect of mucosal extracts from patients with CRS with or without NPs on SIRT1 expression in nasal epithelial cells. Because SIRT1 was highly expressed in epithelium from patients with CRS and lost in NPs (Fig 2, A and B), we hypothesized that features of the mucosal environment could differentially affect epithelial SIRT1 levels, possibly contributing to hypoxia-induced EMT and epithelial remodeling, such as polyp formation. The detailed protocol and concentrations used were described in the [Methods](#) section.

First, RPMI 2650 cells were treated with different mucosal extracts, and then SIRT1 expression was measured (Fig 6, A). Surprisingly, the CRSsNP mucosal extract treatment upregulated the SIRT1 level, but the NP extract treatment did not. Furthermore, the noneosinophilic nasal polyp (NENP) mucosal extract suppressed the SIRT1 level contrary to the eosinophilic nasal polyp (ENP) extract (Fig 6, A).

Next, we determined whether this extract-induced SIRT1 expression could modulate HIF-1 expression and function in primary hNECs (Fig 6, B). Under hypoxic incubation conditions, CRSsNP mucosal extract treatment enhanced the SIRT1 level, which suppressed the expression of HIF-1 α and PDK1. On the contrary, the NP extract-treated cells had a much lower SIRT1 level than CRSsNP-treated cells. Specifically, cells treated with NENP extract had a minimal detectable SIRT1 level and prominent HIF-1 α and PDK1 expression. Interestingly, levels of SIRT1 and HIF-1 α were negatively correlated. In fact, hypoxic incubation with CRSsNP extract did not induce

any mesenchymal morphology in hNECs compared with the other types of extract-treated cells with EMT features (Fig 6, C).

Finally, we measured the transcriptional activity of HIF-1 α with Epo-luciferase reporter plasmids in RPMI 2650 cells under normoxic or hypoxic conditions after treatment with diverse tissue extracts. Similar to HIF-1 α and PDK1 expression, luciferase activity was lower in the CRSsNP extract condition than in control mucosa or in NP extract treatment groups (Fig 6, D, left). Mutated Epo-luciferase activity was not significantly different among the conditions (Fig 6, D, right).

Taken together, our findings suggest that the inflammatory milieu of CRSsNP induces SIRT1, a suppressor of HIF-1 α -mediated epithelial remodeling but that the NP mucosal environment is not conducive to SIRT1 induction.

DISCUSSION

Epithelial remodeling is a characteristic feature of CRSwNP. Specifically, remodeled CRSwNP tissues are frequently characterized by epithelial overgrowth and mesenchymal transition.^{9,20} We reported previously that HIF-1 α -mediated EMT under hypoxic conditions might be an important mechanism involved in NP formation in patients with CRS.⁸ Here we found that SIRT1 suppressed this hypoxia-induced EMT and could inhibit nasal polypogenesis. *In vitro* mechanism studies revealed that SIRT1 activation, which inhibits HIF-1 α transcriptional activity through deacetylation, reversed HIF-1 α -mediated EMT. Immunohistochemical experiments showed that epithelial SIRT1 expression was increased in patients with CRSsNP and diminished or lost in patients with CRSwNP. Thus decreased

SIRT1 expression might boost HIF-1 α -mediated EMT in patients with CRSwNP. Taken together, our findings suggest that SIRT1 activation and maintenance are a plausible means of treating and preventing NP formation in patients with CRS.

Emerging evidence indicates that airway epithelium might contribute to airway remodeling after environmental challenge through the EMT process.²¹ EMT, a reversible process in which epithelial cells transdifferentiate into cells with mesenchymal characteristics, plays a crucial role in body plan formation and in differentiation of multiple tissues and organs.²² Growth factors, as well as inflammatory cytokines and mediators, including TGF- β , are known to weaken epithelial cell-to-cell adhesion and promote mesenchymal marker expression of extra domain A fibronectin, vimentin, and α -SMA.^{21,23} EMT not only plays a crucial role in liver and kidney fibrosis, as well as in the asthmatic lung, but also plays an important role in patients with CRSwNP.^{20,24} Epithelial dedifferentiation toward a mesenchymal phenotype was correlated with airway fibrosis and inflammation.²⁰

In addition to TGF- β , hypoxia is another major EMT-inducing stimulus through HIF-1 activation.^{8,25} In particular, HIF-1 is a known key regulator of cellular responses to low oxygen status by transactivating various downstream genes. Human NPs have higher HIF-1 expression than control nasal mucosa,^{26,27} which reportedly contributes to the epithelial remodeling seen in patients with CRSwNP in diverse ways. For example, VEGF facilitates NP fibroblasts and leads to mucosal edema.^{7,28} HIF-1 α is also a transcription factor that induces expression of a number of EMT-associated genes, such as *TGFB*, *TWIST*, and lysyl oxidase (*LOX*).²⁹ In a mouse model HIF-1 inhibitors suppressed polyp burden and EMT in diverse cells.⁸ In addition, proprotein convertase 1/3 overexpression induced morphological and phenotypic EMT changes in airway epithelial cells, and these changes might have contributed to the pathogenesis of NPs.³⁰

Sirtuins are highly conserved nicotinamide adenine dinucleotide-dependent deacetylases that regulate lifespan in lower organisms³¹ and affect aging-related pathology in mammals, such as diabetes, inflammation, and neurodegenerative disease.³² In mammals there are 7 sirtuin homologs, SIRT1 to SIRT7. Among them, SIRT1 has garnered the most attention. SIRT1 is known to deacetylate transcription factors that control the aging process³³ and suppress inflammation.^{34,35} SIRT1 deacetylates key histone residues involved in the regulation of transcription, including histone H3 lysine 9 (H3-K9), H4-K16, and H1-K26, and diverse nonhistone protein targets, including p53, forkhead box protein O1/3, peroxisome proliferator-activated receptor γ coactivator 1 α , nuclear factor κ B (NF- κ B), and HIFs.^{12,36}

In this study we showed that SIRT1 inhibited hypoxia-induced EMT in nasal mucosa. To our knowledge, this is the first report regarding the role of SIRT1 in nasal polypogenesis. Despite the lack of related literature, a few reports support our findings. Recently, it was shown that SIRT1 suppressed EMT in cancer metastasis and organ fibrosis.^{37,38} In addition, it was previously reported that resveratrol could be a novel drug against CRSwNP because of its action through the 5-lipoxygenase pathway.³⁹ Expression levels of the *IL4*, *IL5*, prostaglandin D synthase, and leukotriene C₄ synthase genes were significantly decreased by administration of low- or high-dose resveratrol.³⁹ However, in these studies the specific mechanism related to the effect of SIRT1 could not be elucidated because of systemic administration of resveratrol, which is also known to suppress inflammation by targeting peroxisome proliferator-activated receptor γ and NF-

κ B.⁴⁰ Given that SIRT1 is a critical negative regulator of both the innate and adaptive immune response in mice,⁴¹ the effect of SIRT1 modulators should be investigated by using site-specific treatment to exclude the systemic effect of SIRT1-targeting drugs. For this reason, we treated with SIRT1 modulators, as well as lentiviral vectors, intranasally and confirmed the antipolyp effect of SIRT1 *in vitro* and *in vivo*.

Interestingly, mucosal extracts from patients with CRSwNP or CRSsNP revealed differential SIRT1 expression in hNECs. CRSsNP mucosal extract induced SIRT1 expression, but NP extract did not. Specifically, NENPs suppressed SIRT1 expression compared with control values, indicating that a different inflammatory milieu in patients could influence SIRT1 levels. Although we observed differential SIRT1 levels after treatment with different mucosal extracts, the underlying mechanism involved in extract-induced SIRT1 induction or suppression is still unknown. Thus far, the regulation mechanism of SIRT1 expression is not fully understood. It has been reported that the SIRT1 protein could be suppressed by the tumor repressor HIC1 (hypermethylated in cancer 1) and the microRNA miR-34a.^{42,43} It is noteworthy that the NF- κ B complex could bind to the promoter of miR-34a, leading to SIRT1 downregulation.⁴⁴ Recently, Li et al⁴⁵ demonstrated that IFN- γ , which is stimulated by NF- κ B signaling, could suppress expression of SIRT1 in skeletal muscle cells. Our preliminary data also showed the possibility that IFN- γ might suppress SIRT1 and boost HIF-1 α activity under hypoxic conditions (see Fig E2). Given that the mucosal environment of NENPs is characterized as higher IFN- γ and IL-17A rather than IL-5 levels, the distinct cytokine milieu might contribute to differential responses against the mucosal extracts of ENPs or NENPs. To further identify the relevance of the inflammatory type of polyp to SIRT1 regulation, we investigated SIRT1, HIF-1 α , and E-cadherin expression between ENP and NENP tissues (see Fig E5 in this article's Online Repository at www.jacionline.org). Although the effect of NP extract on SIRT1 expression was different according to the inflammatory types, there was no significant difference in SIRT1, HIF-1 α , and E-cadherin on immunohistochemistry staining. This was possibly due to the fact that the eosinophilic polyp extracts also could suppress SIRT1 expression and therefore the difference of effects could not be seen. Currently, we could not determine exactly whether SIRT1 suppression is dominant in NENPs rather than ENPs. Further studies are required to clarify this characteristic feature and elucidate the underlying reason.

Taken together, SIRT1 might play a defensive role in CRS, and it seems that SIRT1 loss aggravates sinonasal mucosa inflammation, finally leading to epithelial remodeling, including polypogenesis (Fig 7). Our findings suggest that SIRT1 might be a reliable target for treating nasal polypogenesis through EMT suppression in mucosal epithelium. Moreover, SIRT1 activators could be a novel class of antipolyp drug that modulates HIF-1 activity more delicately than HIF-1 inhibitors. In addition, further investigation regarding the reason underlying loss of SIRT1 expression in NPs could provide a molecular target for novel treatment strategies for nasal polyposis.

Clinical implications: Loss of SIRT1 is found in NPs and accelerates hypoxia-induced EMT. Activating SIRT1 reduces nasal polypogenesis in an animal model and might represent a novel therapeutic target.

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METHODS

Generation of SIRT1 transgenic mice

Transgenic mice overexpressing human SIRT1 were produced by using modified pcDNA3.1 vector containing the myc-tagged SIRT1 with the ubiquitously expressed CMV promoter. Purified transgenic construct DNA was microinjected into fertilized eggs collected from the superovulated C57BL/6 females. Genotyping was performed by means of PCR analysis of genomic DNA obtained from the tails of founder mice at 3 weeks of age, and protein expression was confirmed by means of immunoblotting with anti-Sirt1 and anti-His. Each founder was bred in the hemizygous state, and transgenic and nontransgenic littermates were assigned to pair-matched groups for all experiments. Mice were fed normally in the Institute for Experimental Animals of Seoul National University College of Medicine under specific pathogen-free conditions. These experiments were approved by Institutional Animal Care and Use Committee of Seoul National University (SNU-120313-10-1).

Immunoblotting and immunoprecipitation

Proteins were electrophoresed on 8–12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Temecula, Calif). Membranes were incubated sequentially with primary antibodies. The following antibodies were used: Sirt1 from Millipore at 1:2000 dilution; HIF-1 α generated in our laboratory at 1:1000 dilution^{E1}; PDK1 from Stressgen Biotechnologies (San Diego, Calif) at 1:2000 dilution; vimentin at 1:2000 dilution, β -catenin from Santa Cruz Biotechnology at 1:2000 dilution and β -tubulin from Santa Cruz Biotechnology at 1:5000 dilution; E-cadherin from BD (San Jose, Calif) at 1:2000 dilution; and α -SMA from Abcam (Cambridge, United Kingdom) at 1:2000 dilution. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 hour at room temperature and visualized by using ECL Plus or ECL Select detection reagents (GE Healthcare, Fairfield, Conn). Immunoblotting for β -tubulin served as a protein loading control. For immunoprecipitation, cell lysates were incubated sequentially with 1 μ g of antibodies and 20 μ L of protein A/G sepharose beads for 4 hours at 4°C (Santa Cruz Biotechnologies). Then immunoprecipitates were eluted with a sample buffer (1% SDS, 100 mmol/L dithiothreitol, and 50 mmol/L Tris, pH 7.5) and then subjected to immunoblotting.

Plasmid DNA and small hairpin RNA lentiviral vector transfection

Cells at 60% confluence were transfected with Myc-His-SIRT1 plasmid by using Lipofectamine 2000 reagent (Invitrogen) or small interfering RNAs with RNAi-MAX reagent (Invitrogen). The Myc-His-SIRT1 plasmid was provided kindly by Dr Junjie Chen (University of Texas). For gene silencing, the pLKO.1-puro vector was purchased from Sigma-Aldrich, and oligonucleotides targeting the green fluorescent protein (control small hairpin RNA) or SIRT1 were inserted into the vector by using *Age*I and *Eco*RI restriction enzymes. For SIRT1, 2 oligos, 5'-CCGGAAGTACAACTTC TAGGAATGCTCGAGCATTCCTAGAAAGTTGTACTTTT-3' and 5'-AATTAAGTACAACTTCCTAGGAATGCTCGAGCATTCCTA GAAGTTGTACTT-3', were annealed and ligated into pLKO.1-puro vector to generate sh-SIRT1 RNA, according to the manufacturer's instructions. The construct was confirmed by means of sequencing. The viral vector was cotransfected with pMD2-VSVG, pRSV-RRE, and pMDLg/pRRE helper DNA into HEK293T cells, and the viral supernatant was collected.

Histologic analysis of animal study

The mice were killed and decapitated. Tissue specimens were fixed in 4% paraformaldehyde at 4°C for 1 day and decalcified in 5% nitric acid at 4°C for 3 days. The specimens were excised from the second palatal ridge to the first upper molar teeth. The tissue was dehydrated and processed according to standard paraffin-embedding procedures. The tissue was cut into coronal

sections with a thickness of 4 μ m. Hematoxylin and eosin staining was performed to determine the formation of NPs and eosinophilic infiltration.

An atlas of normal murine sinonasal anatomy was used to standardize the anatomic locations being examined. Scoring of mucosal lesions, including polyps, and epithelial disruption and eosinophilic infiltration were performed under the high-power field (HPF; $\times 400$ magnification) of a microscope by 2 independent examiners blinded to the experimental groups. In case of disagreement (the 2 counts differ by $>10\%$), a consensus was reached by reviewing the specimen at a multihead microscope by our research team. We reviewed 3 consecutive slides to rule out processing errors and confirm mucosal lesions. Polyp formation and epithelial disruption were counted microscopically from 3 coronal sections and expressed as a total number. The epithelial disruption was counted only if epithelial pieces greater than 50 μ m in length stripped off from the subepithelial regions or split from adjacent epithelial cells.

Immunohistochemistry

Sinonasal tissues were fixed in 4% paraformaldehyde at 4°C for 24 hours and then in ethanol and sequentially xylene before embedding them in paraffin. Paraffin sections (4 μ m) of sinonasal tissues were mounted on slides and dried at room temperature for 24 hours. The sections were rehydrated, and autoclaved at 121°C for 10 minutes in 100 mmol/L citrate buffer (pH 6.0; Dako, Glostrup, Denmark) to retrieve antigens. After treatment with 3% hydrogen peroxide, which was diluted in methanol for 10 minutes, the sections were incubated in 2% bovine serum at room temperatures for 1 hour to block nonspecific signals. They were incubated with antibodies against HIF-1 α (1:50; Novus Biologicals, Littleton, Colo), SIRT1 (1:50; Novus Biologicals), and E-cadherin (1:200; BD) overnight at 4°C. Biotinylated secondary antibodies (Vector laboratories, Burlingame, Calif) were used for staining HIF-1 α (1:200), SIRT1 (1:200), and E-cadherin (1:500). The immune complexes were visualized with the Vectastain ABC kit (Vector Laboratories). Negative controls were performed with IgG isotype antibodies (eBioscience, San Diego, Calif). The incubation time for diaminobenzidine staining was fixed in all experiments to standardize color development. All immunostained sections were lightly counterstained with hematoxylin (Mayer, Sigma Aldrich). The slides were evaluated with a bright-field microscope (BX-51; Olympus, Tokyo, Japan) equipped with a camera (DP70, Olympus) and a micrograph field of view of the entire stained section. To score expression levels of E-cadherin, we observed 3 separated HPFs per slide. Detailed methods were described previously.^{E2} Shortly, 3 different spots were randomly selected in every HPF, and 2 independent examiners blinded to the experimental group determined whether E-cadherin was expressed in each spot. The area was defined as a positive spot if more than 30 of the E-cadherin-positive epithelial cells existed continuously. The final score of each sample is presented as the average of scores from 3 HPFs. If the examiners had a disagreement, a consensus was reached by reviewing the specimen at a multihead microscope by our research team. Then the expression level was presented as the mean values of positive spots per HPF. When it comes to the expression of HIF-1 α and SIRT1, the numbers of HIF-1 α - or SIRT1-positive cells were counted in at least 3 different HPFs ($\times 400$ magnification) and presented as mean values.

Human tissue extracts

Freshly obtained tissue specimens were weighed, and all of the tissue volumes are determined equally (110 μ L). One milliliter of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich) and 1% protease inhibitor cocktail (P8340, Sigma-Aldrich) was added for each tissues. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 48°C. After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 48°C, and the supernatants were stored at -70°C until analysis. The total protein concentrations in each of the extracts were measured by using the Bradford assay. Then we used the extract at the adequate protein concentration (20 μ g/mL).

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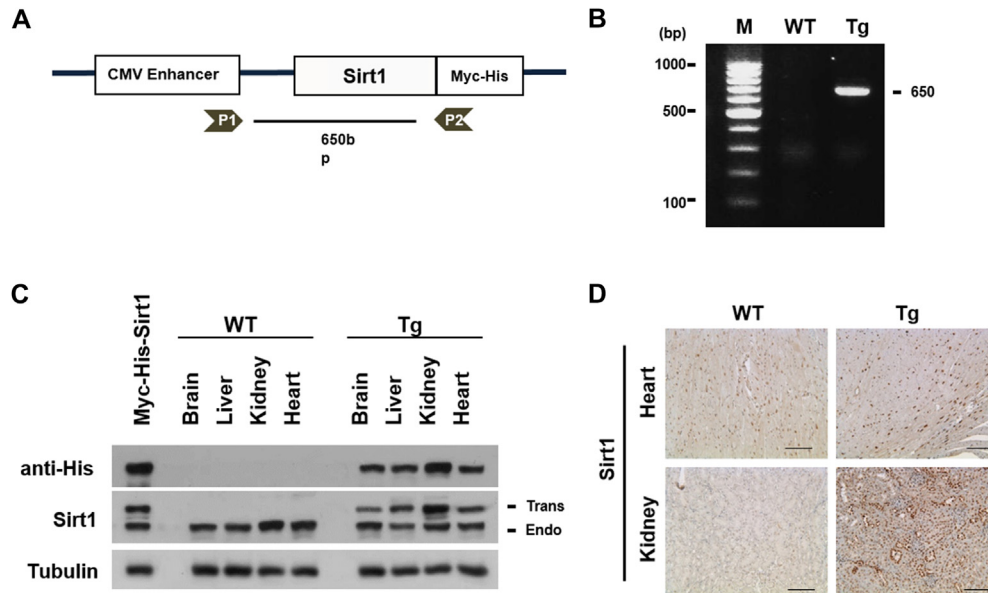


FIG E1. Establishment of the SIRT1 transgenic mouse. **A**, Structure of the Myc/His-tagged SIRT1 expression vector introduced into transgenic mice. Gene expression was driven under the control of a cytomegalovirus (CMV) promoter. **B**, The presence of the SIRT1 vector in transgenic mouse was verified by using PCR. **C**, Expression of endogenous SIRT1 (*Endo*) and transgenic Myc/His-tagged SIRT1 (transgenic [*Tg*]) in WT and SIRT1 transgenic mice. Tissue homogenates were prepared from the brain, liver, kidney, and heart and analyzed by means of Western blotting with anti-His and anti-SIRT1 antibodies. **D**, Paraffin sections of hearts (*top panel*) and kidneys (*bottom panel*) from WT and Sirt1 transgenic mice were subjected to immunohistochemical analyses with anti-SIRT1 antibody. Scale bar = 100 μ m.

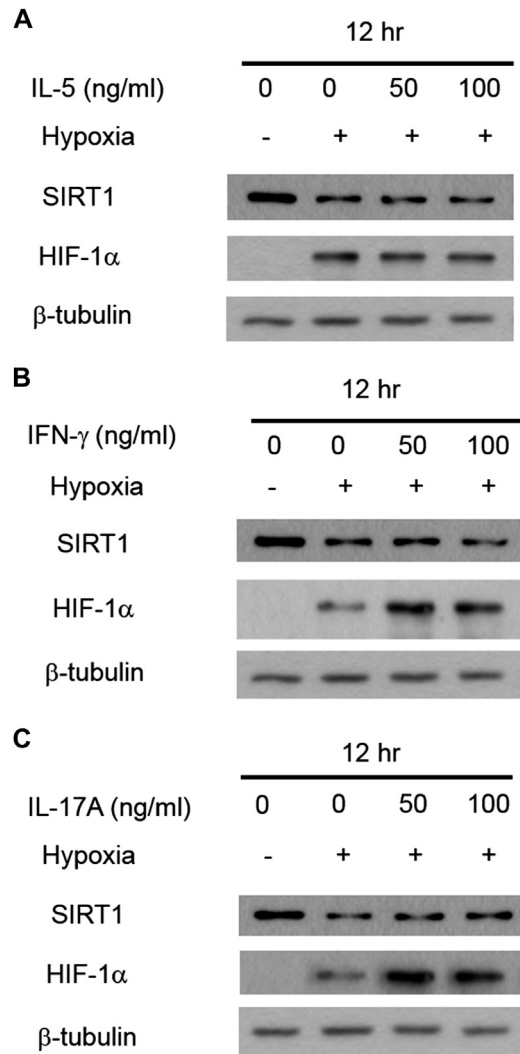


FIG E2. Effect of different inflammatory cytokines on SIRT1 and HIF-1 α expression in nasal epithelial cells. RPMI 2650 cells were cultured under normoxic or hypoxic conditions for 12 hours with the indicated cytokines: **A**, IL-5; **B**, IFN- γ ; **C**, and IL-17A. Cell lysates were prepared for immunoblotting with the indicated antibodies.

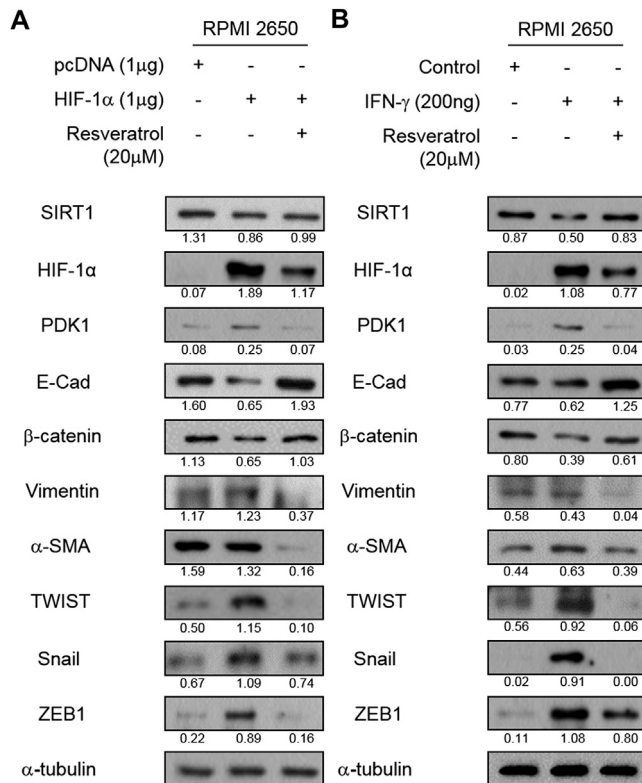


FIG E3. The effect of resveratrol on HIF-1α-induced EMT. **A**, HIF-1α was induced by the transfection of HIF-1α plasmid in RPMI2650 cells, and EMT-related markers were traced after resveratrol treatment. **B**, IFN-γ was treated to RPMI 2650 cells and the effects of resveratrol on EMT-related markers were investigated.

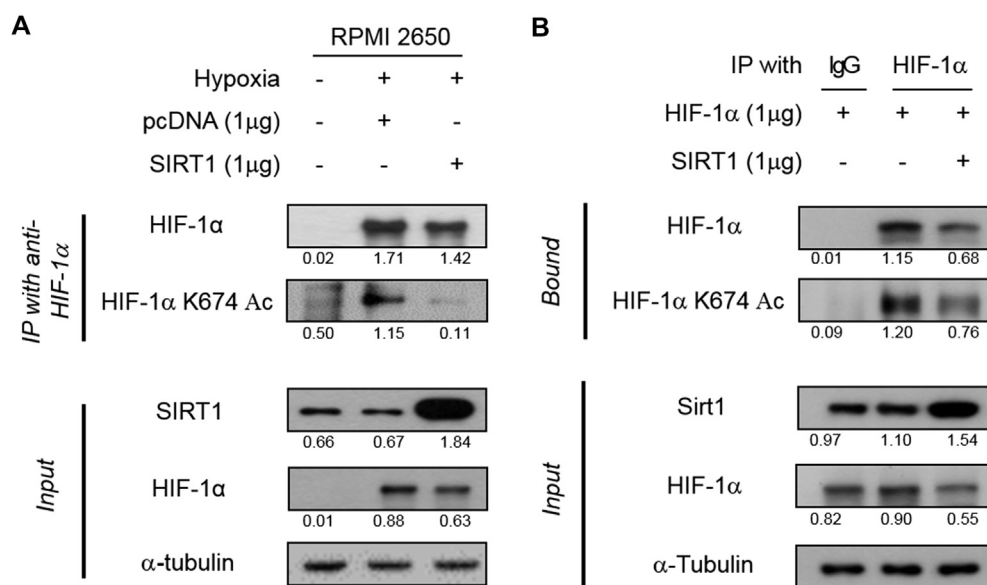


FIG E4. Effect of SIRT1 on HIF-1α deacetylation. **A**, RPMI 2650 cells were transfected with pcDNA or SIRT1 plasmids, followed by 24 hours of normoxic or hypoxic incubation. Whole cell lysates and immunoprecipitated proteins were immunoblotted with the indicated antibodies. **B**, RPMI 2650 cells were transfected with HIF-1α and SIRT1 plasmids, followed by 24 hours of normoxic incubation. Whole cell lysates were treated as mentioned above equally.

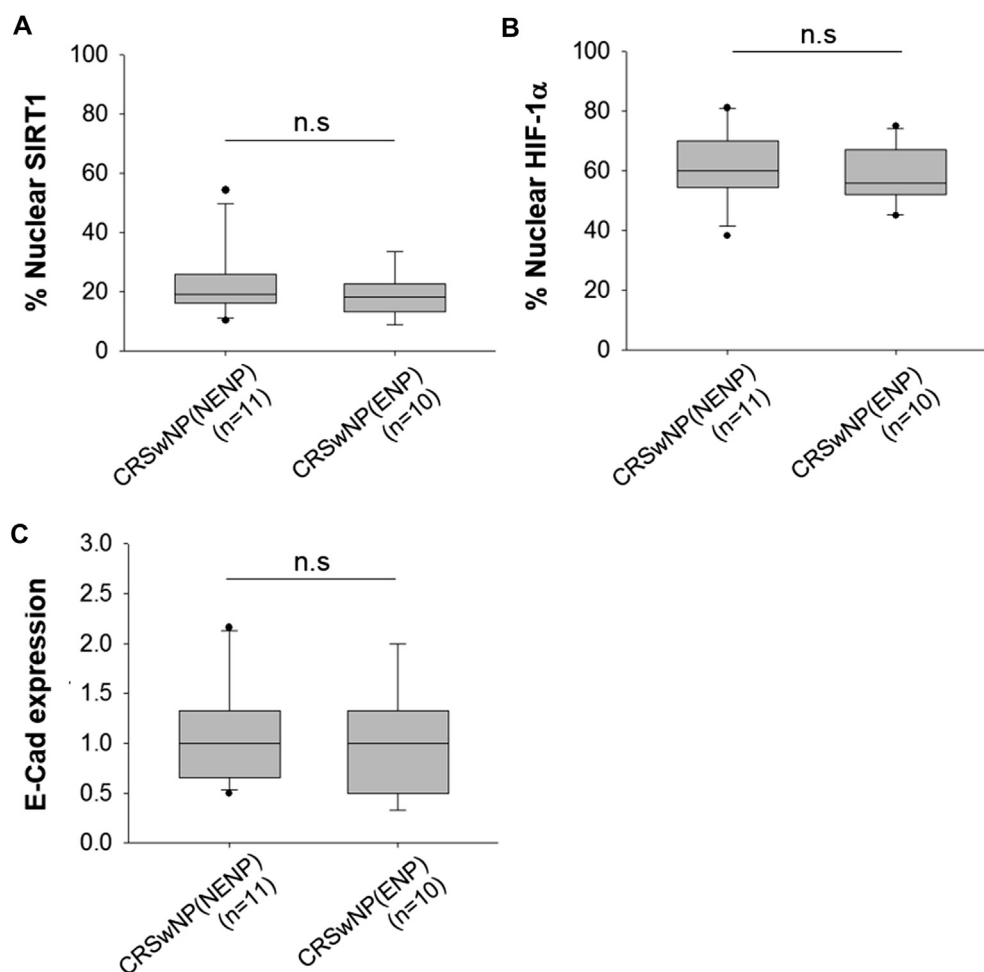


FIG E5. Comparison of SIRT1, HIF-1 α , and E-cadherin (*E-Cad*) expression between ENPs and NENPs of patients with CRS. NP tissues from patients with CRSwNP were immunostained with SIRT1 (**A**), HIF-1 α (**B**), and E-cadherin (*E-Cad*; **C**) antibodies. Comparison of nuclear HIF-1 α , SIRT1, and E-cadherin expression in ENPs and NENPs of patients with CRS. E-cadherin expression was examined with an HPF ($\times 400$ magnification) and scored from 0 to +3. The final score of each sample is presented as the average of scores from 3 HPFs. The detailed criteria for scoring are described in the [Methods](#) section. *n.s.*, Not significant as determined by using the Mann-Whitney *U* test.