Oxidative–protective effect of nuclear receptor coactivator 7 on arecoline-induced endothelial-to-mesenchymal transition

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Objective. Overproduction of reactive oxygen species (ROS) has been implicated in inflammatory activities and tumorigenesis in oral submucous fibrosis (OSF). Nuclear receptor coactivator 7 (NCOA7) is capable of regulating cellular responses to ROS. The aim of this study was to investigate the expression of NCOA7 in endothelial cells and the role of NCOA7 in areca nut–induced endothelial-to-mesenchymal transition (EndMT).

Study Design. Immunohistochemistry and immunofluorescence were used to detect the expression of NCOA7 in endothelia. Human umbilical vein endothelial cells (HUVECs) were treated with various dosages of arecoline (0, 5, 10, 20 μg/mL); then NCOA7 expression, the correlation of NCOA7 with EndMT, and the potential signaling were analyzed by using small interfering RNA (siRNA) transfection, reverse transcription polymerase chain reaction, Western blotting, and flow cytometry.

Results. NCOA7 was significantly elevated in OSF tissues, as detected with immunohistochemistry and immunofluorescence. After arecoline treatment, NCOA7 expression and EndMT were induced in HUVECs. Transfection of HUVECs with si-NCOA7, which reduced 73% of NCOA7 expression, aggravated the arecoline-induced EndMT process. Inhibition of ROS markedly, but not completely, reverses this arecoline-induced EndMT in si-NCOA7 cells.

Conclusions. This study highlights NCOA7 as a potential target for therapeutic intervention to mediate EndMT via ROS species production. (Oral Surg Oral Med Oral Pathol Oral Radiol 2020;130:565–573)

Oral submucous fibrosis (OSF) is a premalignant fibrotic disease affecting the oral mucosa, the oropharynx, and the upper two-thirds of the esophagus. Its malignant transformation rate varies from 1.99% to 9.13%.[1-3] This fibrotic condition is characterized by diffuse, irregular white lesions, which may comprise white marbling and cause progressive inability to open the mouth as a result of reduced blood flow and collagen deposition.[4] The habit of chewing areca nut has been proposed to be the primary etiologic factor in the development of OSF.[5,6] The extract of areca nut contains polyphenols and alkaloids, which can drive the transition of myofibroblasts and the modulation of collagen metabolism.[7]

The fibroblast activation driven by epithelial cells is an important causative event for OSF.[7] Relatively little is known regarding the involvement of endothelial cells in the OSF process. In areca nut chewers, arecoline, at feasible salivary concentration, is cytotoxic for endothelial cells and induce growth inability, cell cycle arrest, and apoptosis of endothelial cells.[8] Arecoline induces the production of endothelial nitric oxide synthase and reactive oxygen species (ROS) in human umbilical vein endothelial cells (HUVECs).[9,10] Upregulation of mesenchymal markers, including vimentin and alpha-smooth muscle actin (α-SMA), have been reported in the endothelia of OSF.[11] Endothelial-to-mesenchymal transition (EndMT) was reported to occur in many fibrotic diseases.[12,13] However, more studies are needed to clarify whether EndMT contributes to the fibrotic process in OSF.

It has been reported that Yes-associated protein mediates arecoline-induced EndMT by ROS signaling.[11] ROS production emerges as a decisive factor in the transforming growth factor-β (TGF-β)–induced EndMT process.[14] Nuclear receptor coactivator 7 (NCOA7), also known as *ERAP140*, contains a TBC/Lysin Motif Domain/ Catalytic （TLDc) domain and is capable of sensing oxidative stress and regulating cellular responses to oxidative damage.[15] NCOA7 is activated and translocated into the nucleus to stimulate the target genes involved in inflammatory cytokines expression, cell proliferation, and tumorigenesis.[16-18] The aim of this study was to determine whether NCOA7 affected the process of EndMT in OSF. We measured NCOA7- and EndMT-related molecules in arecoline-treated HUVECs and OSF tissues and investigated the role of NCOA7 in arecoline-induced

Statement of Clinical Relevance

Nuclear receptor coactivator 7 (NCOA7) was involved in arecoline-induced endothelial-to-mesenchymal transition, apparently through the modulation of oxidative level. Meanwhile, the increase of reactive oxygen species induced by arecoline may act in a negative feedback loop to promote the expression of NCOA7 in oral submucous fibrosis.

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EndMT. Our study demonstrated that oxidative stress was involved in arecoline-induced EndMT and that knocking down of NCOA7 facilitated the EndMT process via ROS signaling.

MATERIALS AND METHODS

Tissue samples

All human tissues were collected after the approval of Institutional Ethics Committee of the Second Xiangya Hospital, Central South University, and the consent of the patients. Patients with OSF were enrolled in the study. Patients with systemic diseases and those who had received prior treatment for OSF were excluded. Normal tissues were obtained from patients without OSF who underwent third molar extractions. An independent set of 10 normal tissues and 30 OSF tissues were selected by random number table and confirmed via pathologic examination.

Immunohistochemistry and immunofluorescence double staining

The tissues were formalin fixed and paraffin embedded. Antigen retrieval was performed by using 10 mM sodium citrate buffer for 30 minutes at 98˚C. The sections were stained with NCOA7 (ab224481, 1:50 dilution; Abcam, Cambridge, MA) by using a standard avidin-biotin-peroxidase complex method. 3-amino-9-ethylcarbazole (AEC; Dako, Carpinteria, CA) was then used to reveal the staining. Negative controls included serial sections in which either the primary or secondary antibodies were excluded. Positive controls included oral squamous cell carcinoma tissues with known NCOA7 upregulation. For immunofluorescence double staining, paraffin-embedded tissue sections were first deparaffinized with xylene, rehydrated with ethanol, rinsed with phosphate-buffered saline (PBS; Beyotime Biotechnology, Shanghai, China) (2 × 5 minutes), and treated with antigen retrieval buffer. Sections were blocked with normal nonimmune goat serum (Auragene Bioscience, Changsha, China) for 60 minutes at room temperature. After this, sections were rinsed with PBS (2 × 5 minutes) and then incubated with primary antibodies against NCOA7 antibody (ab224481, 1:200 dilution; Abcam, Cambridge, MA) and cluster of differentiation 31(ab24590, 1:100 dilution; Abcam, Cambridge, MA) for 10 hours at 4˚C, followed by rinsing with PBS (5 × 5 minutes). Slides were incubated with horseradish peroxidase−conjugated secondary antibodies (Auragene Bioscience, Changsha, China) for 30 minutes at room temperature, rinsed with PBS (5 × 3 minutes). After this, nuclei were labeled with DAPI (4′,6-diamidino-2-phenylindole) for 3 minutes and rinsed with PBS (3 × 3 minutes), and then the sections were mounted. Antifade mounting medium (Auragene Bioscience, Changsha, China) was added to the sections, and then the microscopic inspection was conduct.

Cell culture and treatment

The HUVEC cell line was kindly provided by Professor Chen Lei from the Center for Medical Research, the Second Xiangya Hospital of Central South University. HUVECs were cultured in Dulbecco's modified Eagle's medium cell culture medium (Gibco, Gaithersburg, MD) with 10 vol% fetal bovine serum (FBS; HyClone, Logan, UT) and maintained at 37˚C with 5% carbon dioxide. To investigate the effect of arecoline (Sigma, St. Louis, MO) on NCOA7 and EndMT, cultured HUVECs were exposed to 0, 5, 10, 20 μg/mL of arecoline for 24 hours. To study the impact of arecoline on ROS generation, HUVECs were treated with 20 μg/mL of arecoline for 12 hours. 50 μm *N*-acetylcysteine (Sigma, St. Louis, MO) was used to reduce ROS production. The investigator was blinded to the treatment groups.

Intracellular ROS detection

The ROS assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). HUVECs were treated with 10 μmol/L DCFH-DA (2',7'-dichlorofluorescin diacetate) in Hanks balanced salt solution (Beyotime Biotechnology, Shanghai, China) for 30 minutes before the end of treatment. Then, the fluorescence of the dichlorofluorescein formed from the oxidation of DCFH-DA by cellular oxidants was measured by flow cytometry (FACS Canto II; Ex: 480 nm, Em: 530 nm; BD Biosciences, San Jose, CA).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from freshly isolated HUVECs by using TRIzol reagent (Invitrogen, Waltham, MA) and then reverse-transcribed into complementary DNA (cDNA). RNA was extracted from freshly isolated HUVECs by using TRIzol (ThermoFisher Scientific, Waltham, MA). RNA samples were reverse-transcribed to cDNA by using a PrimeScript RT reagent kit (Takara, Japan). We performed real-time PCR on a LightCycler 96 instrument with SYBR Green mix (Takara, Japan). The relative transcript levels were calculated by using the comparative threshold cycle (ΔΔCT) method; the results were normalized to the expression of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). Supplemental Table S1 (available at [URL]) lists the PCR primer sequences.

Western blot analysis

We resolved 25 mg of protein per lane through electrophoresis by using 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred it to polyvinylidene fluoride membranes (Sigma, St. Louis,

MO). The membranes were blocked with 5% skim milk (Beyotime Biotechnology, Shanghai, China) in tris-buffered saline−Tween (TBS-T; Tris pH 7.2 = 20 mmol/L; NaCl = 137 mmol/L; and 0.1% (v/v) Tween-20) for 1 hour at room temperature. Blots were probed with antibodies against NCOA7 (ab224481, 1:1000 dilution; Abcam, Cambridge, MA), vascular endothelial cadherin (Abcam, ab33168, 1:1000 dilution), cluster of differentiation 31(ab24590, 1:500 dilution; Abcam, Cambridge, MA), α smooth muscle actin (ab5694, 1:1000 dilution; Abcam, Cambridge, MA) and vimentin (ab92547, 1:1000 dilution; Abcam, Cambridge, MA). After extensive washing, the bands were incubated at room temperature for 1 hour with horseradish peroxidase−conjugated secondary antibodies (Goat Anti-Rabbit IgG H&L, 1:2000 dilution; Goat Anti-Mouse IgG H&L, 1:2000 dilution; Beyotime Institute of Biotechnology, Shanghai, China). Chemiluminescence detection (ECL plus Western Blotting Detection System, Amersham, UK) was used to visualize the protein bands. GADPH was used as the internal control.

Cell transfection

HUVECs were transfected with small interfering RNAs (siRNAs) targeting NCOA7 or negative control siRNA (Suzhou Ribo Life Science Co., Ltd., Kunshan, China) with jetPRIME siRNA transfection reagent (Polyplus, France). Transfection was performed by using and following the manufacturer's protocol: 100,000 to 150,000 cells were seeded per well in 2 mL of growth medium 24 hours before transfection; and 110 pmoles siRNA was diluted into 200 μL of jetPRIME buffer for a final 50 nM concentration per well. Then, 4 μL jetPRIME reagent was added into buffer and incubated for 15 minutes at room temperature. The cells were incubated in fresh HUVEC growth medium with transfection mix for 24 to 48 hours. Normal cells and cells transfected with scramble nonsense siRNA served as controls.

Statistical analysis

All data are represented as mean ± standard deviation. P value less than .05 indicated statistical significance. Student t test was used to analyze significant differences between the 2 groups. One-way analysis of variance (ANOVA) was used for multiple comparisons. Statistical analyses were performed by using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

NCOA7 was elevated in oral submucous fibrosis tissues and arecoline-treated HUVECs

To investigate whether NCOA7 was upregulated in endothelial cells, OSF tissues from 30 patients were compared with normal tissues from 10 patients without OSF. Immunohistochemical staining showed that the OSF samples exhibited increased NCOA7 expression in the nucleus of endothelial cells (Figure 1A). The observation was confirmed by immunofluorescence staining (Figure 1B). The level of cluster of differentiation 31 (CD31) was also measured via immunofluorescence, which showed decreased expression in the membrane and cytoplasm of endothelial cells.

We used HUVECs as a model for examining the effect of arecoline on NCOA7 expression. Endothelial cells were cultured with different dosages of arecoline (0, 5, 10, and 20 μg/mL) for 24 hours. As shown in Figure 1C, arecoline-treated cells exhibited significantly higher NCOA7 protein expression in a dose-dependent manner. Compared with the untreated group, NCOA7 increased about 3.29-, 7.31-, and 10.32-fold after exposure to 5, 10, and 20 μg/mL arecoline, respectively.

Arecoline-induced EndMT of HUVECs

To determine whether arecoline caused EndMT in this fibrotic process, HUVECs were treated with different concentrations of arecoline for 24 hours. Morphologically, the untreated HUVECs were generally polygonal in appearance. After exposure to 20 μg/mL arecoline for 24 hours, the phenotype of HUVECs was altered from a typical cobblestone-like appearance to a fibroblast-like appearance (Figure 2A). Western blot analysis showed downregulation of VE-cadherin ($P < .05$) and CD31 ($P < .05$), which are endothelial-specific markers, in a dose-dependent manner, whereas mesenchymal markers, such as vimentin ($P < .05$) and α-SMA ($P < .05$), were upregulated. These results implied that arecoline could induce mesenchymal transition of HUVECs (Fig. 2B and 2C).

NCOA7 knock-down promoted arecoline-induced EndMT

To examine the association between NCOA7 and this arecoline-induced EndMT, we knocked down NCOA7 with siRNA and observed promotion of arecoline-induced EndMT. After transfection with siRNA, which showed a significant reduction in NCOA7 levels (~73%) at 24 hours (Fig. 3A), HUVECs were challenged with 20 μg/mL arecoline for 48 hours. Knocking down NCOA7 increased mesenchymal marker expressions, including vimentin ($P < .05$) and α-SMA ($P < .05$) and decreased endothelial-specific maker expressions, such as CD 31 ($P < .05$) and VE-cadherin ($P < .05$) at the messenger RNA (mRNA) and protein levels (Fig. 3B and 3C). To avoid off-target effects, one more siRNA targeting another site was used in key experiments (Supplemental Figure S1), and consistent results were obtained. Administration of

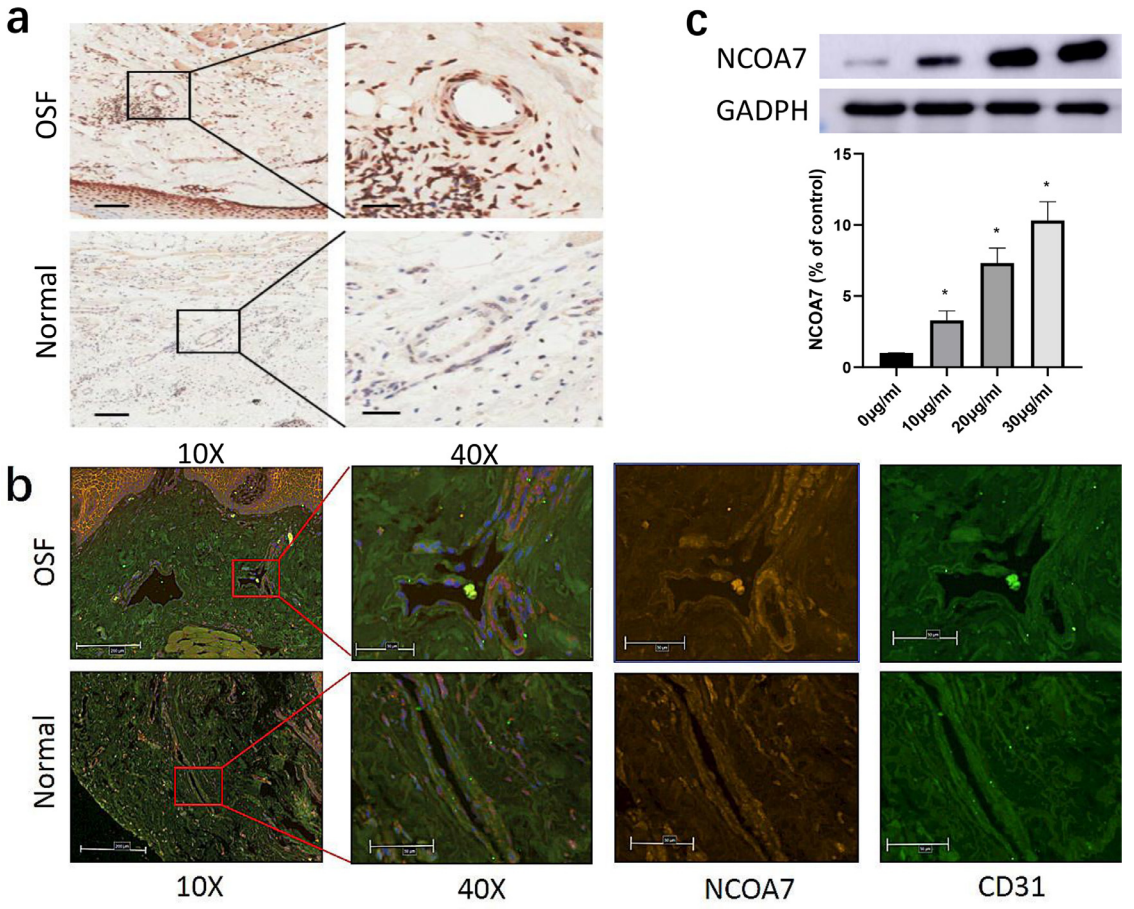


Fig. 1. Expression of NCOA7 in OSF tissues and arecoline treated HUVECs. **A,** Representative images of NCOA7 immunostaining of OSF tissues and normal tissues. (*left,* × 10 magnification; *right,* × 40 magnification). NCOA7 was highly expressed in the nucleus of endothelial cells in OSF samples. **B,** Immunofluorescence double-staining of NCOA7 and CD31 in OSF tissues versus normal tissues. Tissues were stained with antibodies for NCOA7 *(magenta)*, CD31 *(green)*, and DAPI *(blue)*. NCOA7 was increased in the nucleus of endothelial cells, whereas CD31 was decreased in the membrane and cytoplasm of endothelial cells in OSF samples. **C,** Western blotting of NCOA7 in HUVECs treated with 0, 10, 20, 30 μg/mL arecoline. GADPH was performed to monitor equal protein loading, and the 0 μg/mL group was set as 1.0. Results are shown as mean ± SD. Student *t* test. $^*P < .05$. The experiment was repeated 3 times independently. *CD31,* cluster of differentiation 31; *DAPI,* 4′,6-diamidino-2-phenylindole; *GADPH,* glyceraldehyde-3-phosphate dehydrogenase; *HUVEC,* human umbilical vein endothelial cell; *NCOA7,* nuclear receptor coactivator 7; *OSF,* oral submucosa fibrosis; *SD,* standard deviation.

si-NC and transfection reagent did not affect the expression of EndMT-related markers (Fig. 4).

NCOA7 was involved in arecoline-induced ROS production

To investigate the role of NCOA7 in HUVECs during arecoline treatment, the endothelial cells that knock down NCOA7 by siRNA transfection were incubated for 12 hours with 20 μg/mL of arecoline, and ROS production was then determined by flow cytometry and compared with only arecoline-treated HUVECs. As shown in Figure 5, after arecoline treatment, ROS production was increased in the arecoline-treated group, whereas co-treatment with arecoline and si-NCOA7 resulted in an even higher level of ROS. These results suggest that NCOA7 might play a protective role in arecoline-induced ROS production.

N-acetylcysteine reversed arecoline-induced EndMT process in si-NCOA7 HUVECs

To examine whether si-NCOA7 mediated EndMT via ROS signaling, si-NCOA7 HUVECs were pretreated with antioxidant (50 μM) for 2 hours before and during arecoline treatment, and then expressions of EndMT-related factors (α-SMA, vimentin, CD31, and VE-cadherin) were determined via Western blotting (Fig. 6). Compared with group co-treatment with si-NCOA7 and arecoline, expression of α-SMA and vimentin were decreased and the expression of

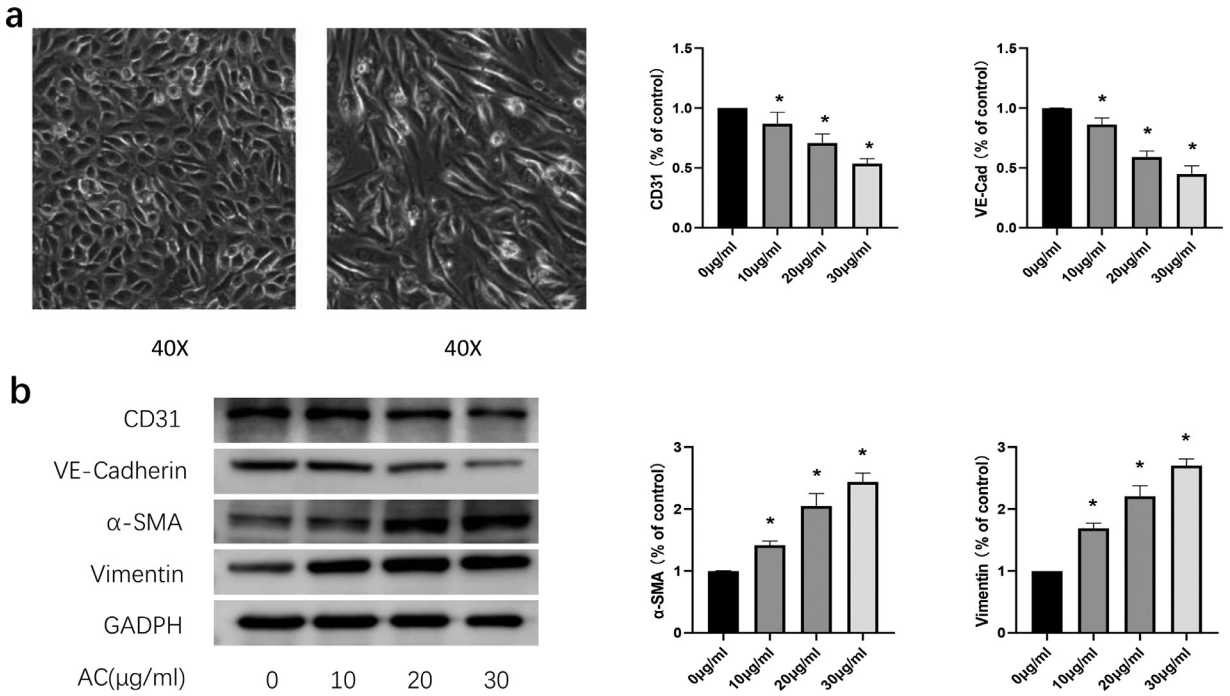


Fig. 2. Arecoline induced endothelial mesenchymal transition of HUVECs. **A,** HUVECs showed fibroblast-like morphologic changes after arecoline treated for 24 hours. **B,** Western blotting of CD31, VE-cadherin, α-SMA, Vimentin in HUVECs treated with 0, 10, 20, 30 μg/mL arecoline. **C,** The relative level of protein expression for each sample was normalized against GADPH signal, and the 0 μg/mL group was set as 1.0. Results are shown as mean $\pm$ SD. Student *t* test. *$P < .05$. The experiment was repeated 3 times independently. *α-SMA,* alpha-smooth muscle actin; *AC,* arecoline; *CD31,* cluster of differentiation 31; *GADPH,* glyceraldehyde-3-phosphate dehydrogenase; *HUVEC,* human umbilical vein endothelial cell; *SD,* standard deviation; *VE-cadherin,* vascular endothelial cadherin.

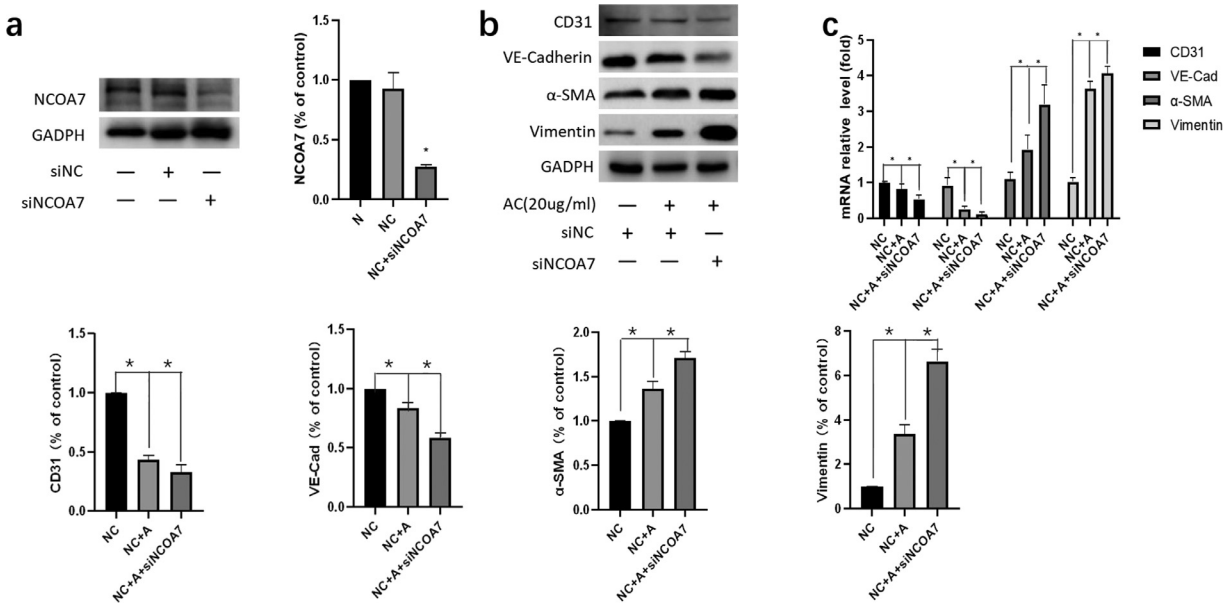


Fig. 3. The effect of NCOA7 knock-down on arecoline-induced endothelial-to mesenchymal transition. **A,** Western blotting of NCOA7 in HUVECs with or without si-NCOA7 transfection. The relative protein level of NCOA7 was normalized against GADPH signal, and the N group (normal HUVECs) was set as 1.0. Results are shown as mean $\pm$ SD. Student *t* test. *$P < .05$. HUVECs with si-NCOA7 significantly reduced ~73% protein expression. **B,C,** Western blotting and RT-PCR of CD31, VE-cadherin, α-SMA, and vimentin in arecoline treated HUVECs with or without si-NCOA7 transfection. The relative level of NCOA7 was normalized against GADPH signal, and the NC group (HUVECs transfected with si-NC) was set as 1.0. Results are shown as mean $\pm$ SD. Analysis of variance (ANOVA). *$P < .05$. *α-SMA,* alpha-smooth muscle actin; *CD31,* cluster of differentiation 31; *GADPH,* glyceraldehyde-3-phosphate dehydrogenase; *HUVEC,* human umbilical vein endothelial cell; *NCOA7,* nuclear receptor coactivator 7; *RT-PCR,* real-time polymerase chain reaction; *SD,* standard deviation; *VE-cadherin,* vascular endothelial cadherin.

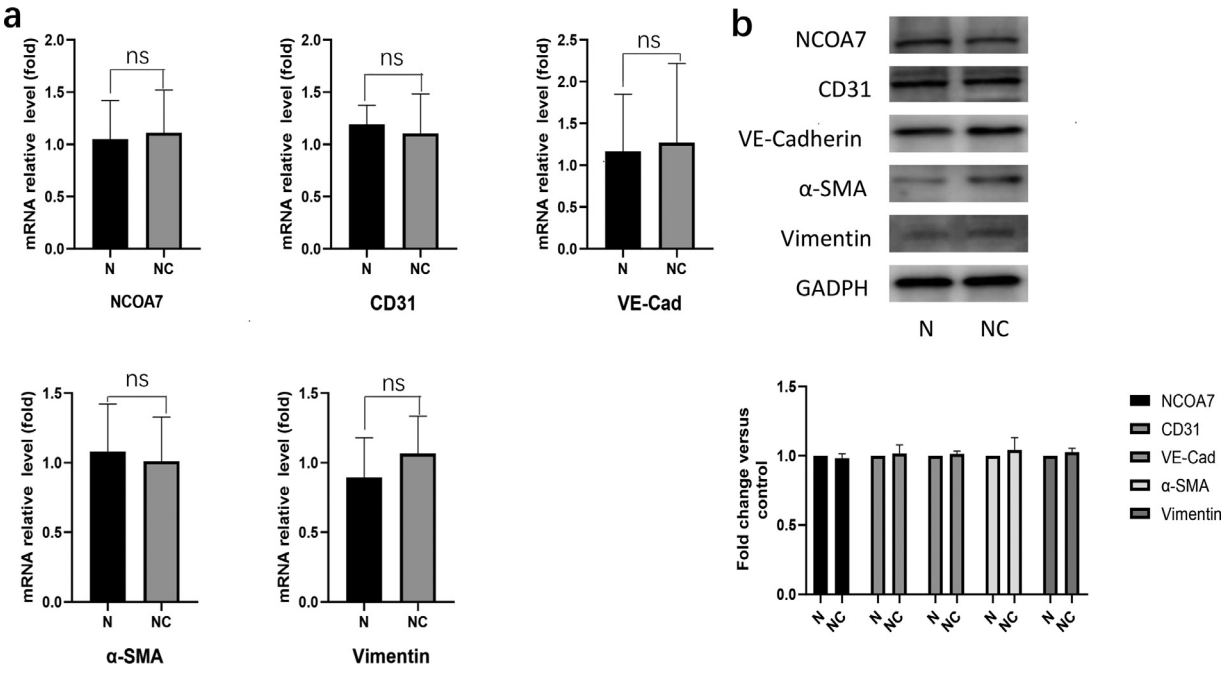
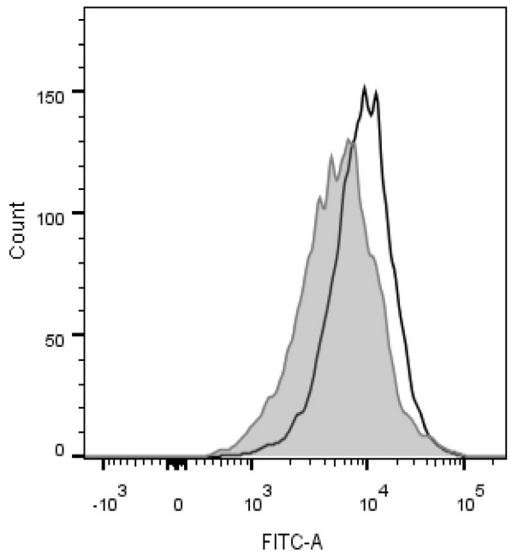


Fig. 4. There were no significant differences between the N and NC groups. N represents normal HUVECs group; NC represents HUVECs with si-NC and transfection reagent. **A,** The mRNA levels of NCOA7, CD31, VE-cadherin, α-SMA, and vimentin were detected by RT-PCR. Results are shown as mean ± SD. Student *t* test. ns represents not statistically significant. **B,** Western blotting of NCOA7, CD31, VE-cadherin, α-SMA, and vimentin in the N and NC groups. The relative levels of protein expression were normalized against GADPH signal, and the 0 μg/mL group was set as 1.0. Results are shown as mean ± SD. Student *t* test. ns represents not statistically significant. The experiment was repeated 3 times independently. *α-SMA,* alpha-smooth muscle actin; *CD31,* cluster of differentiation 31; *GADPH,* glyceraldehyde-3-phosphate dehydrogenase; *HUVEC,* human umbilical vein endothelial cell; *NCOA7,* nuclear receptor coactivator 7; *RT-PCR,* real-time polymerase chain reaction; *SD,* standard deviation; *VE-cadherin,* vascular endothelial cadherin.

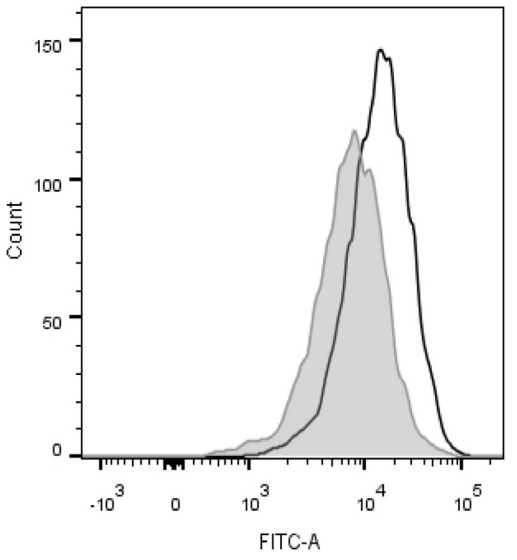
CD31 and VE-cadherin were upregulated, suggesting that *N*-acetylcysteine partly reversed this arecoline-induced EndMT transition in si-NCOA7 cells. Taken together, si-NCOA7 facilitates arecoline-induced EndMT through an oxidative-dependent mechanism.

DISCUSSION

OSF is an inflammatory and progressive fibrotic disease with malignant potential. However, the development of therapies has been limited by incomplete understanding of the pathogenesis and carcinogenesis of OSF. Pathologically, the initial period of fibrosis is marked by microvascular dilation, fibroblasts activation, and extracellular components centered on microvasculature.[19] Our data showed that arecoline promoted EndMT in HUVECs, in agreement with that of Li et al.,[11] who found that EndMT markers, including N-cadherin, vimentin, and α-SMA, were upregulated in endothelia. Although having little direct effect on fibroblasts, arecoline mediates the expressions of interleukin-1 (IL-1), IL-6, colony stimulating factor, and basic fibroblasts growth factor in HUVECs.[18] Arecoline is reported to significantly increase endothelial nitric oxide synthase and ROS expression in HUVECs, mitochondrial membrane potential hyperpolarization in epithelial cells, cyclooxygenase-2 expression in buccal fibroblasts,[10,20,21] suggesting that arecoline can induce ROS production in several ways. Accumulating evidence has indicated that ROS promotes EndMT in various diseases. Montorfano et al. found that oxidative stress was crucial for the conversion of HUVECs into myofibroblasts via the ALK5/Smad3/NF-κB intracellular pathway, supporting the hypothesis of an interaction among ROS signaling, TGF-β signaling, and the EndMT transition process.[14] In line with this observation, lipopolysaccharide-induced ROS promoted TGF-β–related EndMT in vascular endothelial cells.[22] Our finding that EndMT responses (morphologic changes, altered protein expression) after the change of ROS level is in agreement with that of the studies mentioned above.



	Group	Count	Geometric Mean: FITC-A
	NC	7186	5583
	NC+AC	6599	9030



	Group	Count	Geometric Mean: FITC-A
	NC+AC	5774	7411
	NC+AC+Si	6624	13938

Fig. 5. Cells were pretreated with si-NC or si-NCOA7 for 24 hours, then incubated for 12 hours in the presence of arecoline (10 μg/mL). Intracellular ROS production was measured by flow cytometry. *Ac,* presents arecoline; *HUVEC,* human umbilical vein endothelial cell; *NC,* HUVECs with si-NC; *NCOA7,* nuclear receptor coactivator 7; *ROS,* reactive oxygen species; *Si,* NCOA7 knock-down cells.

NCOA7 contains a conserved domain of approximately 200 amino acids called *TLDc*, which is capable of sensing oxidative stress and regulating cellular responses that affect the expression of key oxygen free radical scavengers.[23] In this report, we first demonstrated that NCOA7 is highly expressed in OSF tissues and arecoline-induced HUVECs. Our previous study proved that NCOA7 was upregulated in oral squamous cell carcinoma arising from OSF and that expression of NCOA7 proteins was related to life expectancy.[16] We attempted to evaluate whether NCOA7 regulated oxidative stress in arecoline-exposed HUVECs. We found that 73% knocking down of NCOA7 facilitated ROS accumulation and aggravated arecoline-induced EndMT. This process could be partly reversed by the ROS inhibitor *N*-acetylcysteine. ROS plays both deleterious and beneficial roles in oral mucosal disease.[24] Arecoline-induced ROS generation is involved in cell apoptosis, cell differentiation, and DNA damage.[8,25] Ironically, some ROS-mediated factors protect cells against oxidative damage. Although NCOA7 was upregulated with increased concentrations of arecoline, 75% knocking down of NCOA7 increased the sensitivity of HUVECs to arecoline-induced oxidative stress. Taken together, NCOA7 might be a protective factor against oxidative stress and influence oxidative-dependent EndMT. However, 42% knock-down of NCOA7 did not result in similar effects (see Supplemental Figure S2, available at [URL]). Therefore, there might be a threshold level of NCOA7 expression, below which other cellular mechanisms cannot compensate. Various ROS-mediated actions protect cells from oxidative damage to re-establish or maintain the "redox homeostasis." High ROS expression activates protective mechanisms to maintain redox balance, as in ischemic preconditioning during ischemic injury to the heart. In OSF, arecoline, as a pro-oxidant, promotes ROS generation, and correspondingly, NCOA7 might act as an oxidative protector to maintain redox balance as its expression increases with the increase of ROS. This explains why NCOA7, a protective factor, is highly expressed in OSF.

CONCLUSIONS

Our results suggest that NCOA7 is involved in arecoline-induced EndMT, apparently through the modulation of oxidative level. Meanwhile, the increase of ROS induced by arecoline may act in a negative feedback loop to promote the expression of NCOA7.

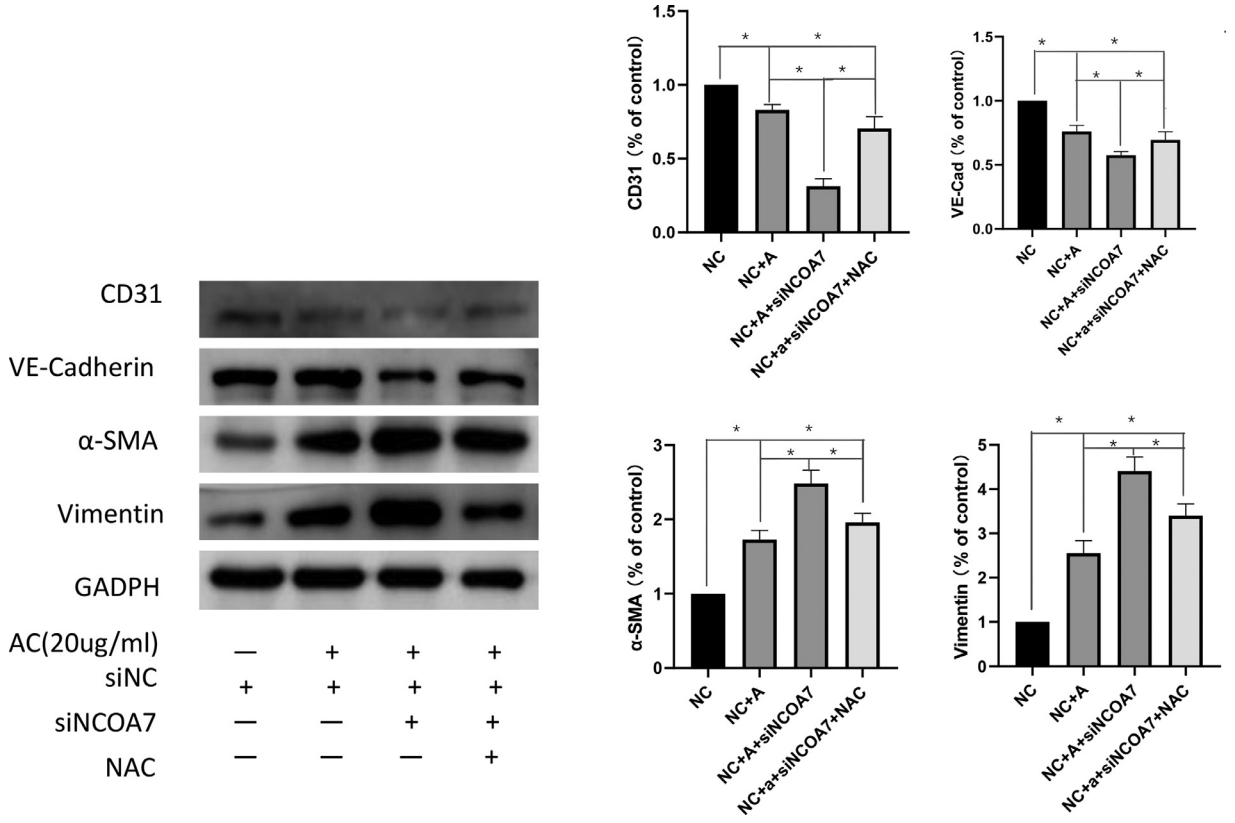


Fig. 6. The effect of *N*-acetylcysteine on endothelial-to mesenchymal transition process in si-NCOA7 cells. HUVECs were treated with N-acetylcysteine (50 μM) for 2 h post si-NCOA7 transfection, then incubated for 24 hours in the presence of arecoline (20 μg/mL). The protein levels of CD31, VE-cadherin, α-SMA, and vimentin were detected by using Western blotting. The relative level of protein expression for each sample was normalized against GADPH signal, and the NC group was set as 1.0. Results are shown as mean ± SD. Analysis of variance (ANOVA). *$P < .05$. The experiment was repeated 3 times independently. Compared with arecoline treated siNCOA7 cells, *N*-acetylcysteine could partly reverse this arecoline-induced endothelial-to-mesenchymal transition process. *α-SMA,* alpha-smooth muscle actin; *CD31,* cluster of differentiation 31; *GADPH,* glyceraldehyde-3-phosphate dehydrogenase; *HUVEC,* human umbilical vein endothelial cell; *NCOA7,* nuclear receptor coactivator 7; *ROS,* reactive oxygen species; *SD,* standard deviation; *VE-cadherin,* vascular endothelial cadherin.

FUNDING

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.oooo.2020.08.018.

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