DEP-induced ZEB2 promotes nasal polyp formation via epithelial-to-mesenchymal transition

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Graphical Abstract

DEP exacerbate CRS and contribute to nasal polyp formation through ZEB2-dependent EMT in hNECs.

Biopsy from: Normal subject
Healthy mucosa
DEP exposure (PM2.5, PM10)
Healthy EMT
Protein Healthy
E-Cad ↓
ZEB2 ↑

Biopsy from: CRSsNP patient
CRS mucosa
DEP exposure (PM2.5, PM10)
Severe EMT
Protein CRSsNP
E-Cad ⊘⊘⊘
ZEB2 ⊘⊘⊘

Abbreviations used: DEP, diesel exhaust particles; CRSsNP, chronic rhinosinusitis without nasal poly; CRSwNP, chronic rhinosinusitis with nasal poly; E-Cad, e-cadherin; EMT, epithelial-mesenchymal-transition; hNEC, human nasal epithelial cells.

Received for publication September 25, 2020; revised April 8, 2021; accepted for publication April 16, 2021. Available online May 4, 2021. Corresponding author: Hyun-Woo Shin, MD, PhD, Department of Pharmacology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea, E-mail: charlie@snu.ac.kr.© 2021 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
https://doi.org/10.1016/j.jaci.2021.04.024
Background: Diesel exhaust particles (DEPs) are associated with the prevalence and exacerbation of allergic respiratory diseases, including allergic rhinitis and allergic asthma. However, DEP-induced mechanistic pathways promoting upper airway disease and their clinical implications remain unclear. Objective: We sought to investigate the mechanisms by which DEP exposure contributes to nasal polyposis using human-derived epithelial cells and a murine nasal poly (NP) model. Methods: Gene set enrichment and weighted gene coexpression network analyses were performed. Cytotoxicity, epithelial-to-mesenchymal transition (EMT) markers, and nasal polyposis were assessed. Effects of DEP exposure on EMT were determined using epithelial cells from normal people or patients with chronic rhinosinusitis with or without NPs. BALB/c mice were exposed to DEP through either a nose-only exposure system or nasal instillation, with or without house dust mite, followed by zinc finger E-box-binding homeobox (ZEB)2 small hairpin RNA delivery.

Results: Bioinformatics analyses revealed that DEP exposure triggered EMT features in airway epithelial cells. Similarly, DEP-exposed human nasal epithelial cells exhibited EMT characteristics, which were dependent on ZEB2 expression. Human nasal epithelial cells derived from patients with chronic rhinosinusitis presented more prominent EMT features after DEP treatment, when compared with those from control subjects and patients with NPs. Coexposure to DEP and house dust mite synergistically increased the number of NPs, epithelial disruptions, and ZEB2 expression. Most importantly, ZEB2 inhibition prevented DEP-induced EMT, thereby alleviating NP formation in mice.

Conclusions: Our data show that DEP facilitated NP formation, possibly via the promotion of ZEB2-induced EMT. ZEB2 may be a therapeutic target for DEP-induced epithelial damage and related airway diseases, including NPs. (J Allergy Clin Immunol 2022;149:340-57.)

Key words: Diesel exhaust particles, air pollutants, nasal polyps, epithelial-to-mesenchymal transition, ZEB2

The global incidence of allergic diseases has markedly increased since the 1960s. This increase is associated with increased environmental factors such as air pollutants, allergens in the air, smoke, and varied genetic background. Among these, the influence of air pollution on human health has become a major global environmental concern impacting the development of allergic diseases. Particulate matter (PM), a major component of air pollutants, was the ninth risk factor among attributable burdens of disease globally and the fourth risk factor in East Asia. Based on previous reports, diesel exhaust particles (DEPs) derived from traffic and industrial activities significantly contribute to urban PM. The exposure to DEP is associated with the exacerbation of respiratory diseases, particularly in larger cities with considerable traffic pollution.

DEPs are composed of a fine carbon core (PM2.5 [PM with aerodynamic diameter <2.5 μm]), a mixture of organic compounds, for example, polycyclic aromatic hydrocarbons, quinones, aldehydes, nitroaranes, and absorbed metals. The effects of DEPs at the cellular level have been extensively studied, and it has been reported that DEPs can upregulate various cytokines and chemokines in airway epithelial cells (AECs) such as IL-6, IL-8, RANTES, and GM-CSF. DEP exposure activates cell cycle progression in A549 cells and induces epithelial-derived innate cytokines (IL-33 and thymic stromal lymphopoietin) at both transcriptional and translational levels in the human bronchial epithelial cells (HBECs). In experimental animal studies, administration of DEPs along with an allergen increased TH2 inflammation and IgE secretion specific to the allergen. The adjuvant effect of DEPs on IgE production has also been revealed. It has been demonstrated that DEPs aggravate allergic rhinitis (AR) by disrupting tight junction protein. Bioinformatics studies showed that the genes changed by the DEP treatment in HBECs are involved in inflammatory response and epithelial-to-mesenchymal transition (EMT). Epidemiological studies exhibited the positive correlation between traffic-related air pollution and AR symptoms. EMT is characterized by progressive loss of epithelial features, including apicobasal polarity disruption, cell-to-cell junctional loss, and epithelial layer integrity impairment, and finally, transdifferentiation into mesenchymal cells by continuous gain of mesenchymal features, such as acquisition of front-back polarity, activation of EMT-triggering transcription factors, and expression of mesenchymal markers. Three types of EMT have been reported, which are associated with embryonic gastrulation, wound healing and repair, and cancer cell metastasis, respectively. In particular, repetitive occurrence of type 2 EMT, which is associated with tissue regeneration and fibrosis by inflammation-inducing injuries, is considered an important feature that is observed in respiratory airway diseases, including asthma and chronic rhinosinusitis (CRS) with nasal polyps (NPs) (CRSsNP) or without NPs (CRSsNP). Multiple types of transcripts, such as CDH1, CDH2, ACTA2, and VIM, are required for
EMT progression via transcriptional reprogramming mediated by the zinc finger E-box-binding homeobox (ZEB) family (ZEB1 and ZEB2/SIP1), the Snail family (Snail1, Snail2/Slug, and Snail3), and the TWIST family (Twist1 and Twist2). Although ZEB2, a master regulator of EMT, has been extensively studied in various types of disease, our understanding of the clinical importance of ZEB2 in terms of airway disease remains poor.

Although many of these studies have reported that DEP exposure could influence the prevalence of upper airway diseases, the molecular mechanisms responsible for the DEP-induced upper airway diseases, to our knowledge, still remain unclear. In addition, most studies using DEP have been limited to animals or performed in vitro using cell cultures, indicating the necessity of further investigation, which could link the effect of DEP and clinical implications.

In the present study, we investigated the effect of DEP exposure on human nasal epithelial cells (hNECs) using bioinformatics analyses and various in vitro and in vivo experiments, and explored ZEB2-induced EMT in the context of NP formation, a chronic upper airway disease, to understand and elucidate the clinical implications for potential therapeutic avenues.

METHODS

Study participants and characteristics

The Institutional Review Board of Seoul National University Hospital approved this study (approval C-1902-098-1011), and all subjects provided written informed consent. Subjects with control, CRSsNP, and CRSwNP were recruited from the Department of Otolaryngology clinics at the Seoul National University School of medicine. Details of the participant characteristics are summarized in Table I. The diagnosis of sinusitis or nasal polyposis was determined in subjects with CRS by personal medical history, physical examination, nasal endoscopy, and computed tomography findings of the sinuses following the European position paper on rhinosinusitis and nasal polyps 2012 guidelines. Uncinate process tissues and NP tissues were collected at the time of routine endoscopic sinus surgery from patients with CRSwNP or CRSsNP. Normal inferior turbinate mucosal tissues were used as control, which were obtained from the patients who underwent nasal septoplasty surgery. All control subjects had no history of sinonasal inflammation, asthma, or use of steroid. We excluded the patients with any of followings: (1) younger than 18 years; (2) unstable asthma symptoms, asthmatic or aspirin-sensitive patients; (3) use of antibiotics, inhaled systemic or topical corticosteroids, or other immune-modulating drugs up to 4 weeks before surgery; and (4) suffered from conditions such as unilateral rhinosinusitis or unilateral NPs, anticoagulant polyphs, cystic fibrosis, immotile ciliary disease, a systemic coagulation disorder, and immunodeficiency. The atopic status of study patients and the diagnosis of asthma were evaluated as previously described. Lund-Mackay computed tomography scores were obtained before surgery.

Cell line and cell culture

The LA4 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI-1640 medium (Welgene, Seoul, Korea) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin in a 5% CO₂ humidified incubator at 37°C following the supplier’s recommendations.

Isolation and culture of primary hNECs

Primary hNECs of normal donors were purchased from Promocell (Heidelberg, Germany). Cells were incubated as monolayers in an airway epithelial cell growth medium (PromoCell) supplemented with the SupplementMix (PromoCell), cultured in a 75-cm² T-flask in a humidified incubator at 37°C in 5% CO₂. For air-liquid interface (ALI) culture, primary hNECs were isolated from tissue specimens of control subjects, patients with CRS, and patients with CRSwNP. Nasal samples were obtained from the inferior turbinate of control subjects and from the uncinated process and accessible polyp tissues in subjects with CRSsNP and CRSwNP during surgery. The tissues were digested enzymatically by incubating overnight in a 1:1 mixture of Dulbecco modified Eagle medium (DMEM), Ham’s nutrient Mix (DMEM/F12) added with penicillin (200 U/mL) and streptomycin (200 μg/mL) containing 0.1% Pronase (type XIV protease) (Sigma-Aldrich, St. Louis, Mo). After incubation, nasal epithelial cells were detached from the stroma by gentle agitation and mechanical injury using a yellow pipette tip, and were centrifuged at 200g for 5 minutes. The pellet was suspended in DMEM (Lonza, Walkersville, Md) containing 10% FBS and was centrifuged again. The second pellet was resuspended with bronchial epithelium basal medium (Lonza) supplemented with bronchial epithelial growth medium (BEGM) SingleQuot Kit Supplements (Lonza) and plated on plastic culture dishes. The culture media was refreshed on day 1 after seeding, and every second day thereafter until cells reached 60% to 70% confluence in a humidified atmosphere of 5% CO₂ at 37°C. Cells were detached with trypsin-EDTA 0.05% (Gibco, Carlsbad, Calif). Then, cell numbers were decided by hemocytometer. The detailed culture methods have previously been described.

ALL culture system

hNECs, expanded once or twice with growth medium, were seeded onto 0.4-μm, 0.33-cm² polyester Transwell inserts (Corning Inc, Corning, NY) at a density of 1 × 10⁶ cells per well. Cells were grown submerged for 5 to 7 days to complete confluence using a 1:1 mixture of BEGM (Lonza) supplemented with BEGM SingleQuot Kit Supplements (Lonza) and DMEM (Lonza). The culture medium was replaced on day 1 and refreshed every other day. Once the cells have reached confluence (usually 5-7 days), the ALL was created by removing the medium from the apical compartment. Cells were maintained

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TABLE I. Patient characteristics and methodologies used

<table>
<thead>
<tr>
<th>Characteristic and methodology</th>
<th>Control (N = 10)</th>
<th>CRSsNP (N = 7)</th>
<th>CRSwNP (N = 7)</th>
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<tr>
<td>Tissue used</td>
<td>InfTurb</td>
<td>InfTurb</td>
<td>NP</td>
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<td>Age (y), mean ± SD</td>
<td>51.7 ± 17.1</td>
<td>42.4 ± 18.8</td>
<td>53.7 ± 14.6</td>
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<tr>
<td>Asthma, N</td>
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<td>0</td>
</tr>
<tr>
<td>Aspirin intolerance, N</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nasal steroid, N</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Oral steroid, N</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lund-Mackay CT score</td>
<td>1.4 (2.06)</td>
<td>10.14 (4.4)</td>
<td>12.42 (4.6)</td>
</tr>
<tr>
<td>Blood eosinophils (%) (SD)</td>
<td>2.4 ± 1.4</td>
<td>3.7 ± 3.3</td>
<td>2 ± 1.76</td>
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<td>Methodologies used</td>
<td></td>
<td></td>
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<tr>
<td>RNA sequencing</td>
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<td>N = 0</td>
<td>N = 0</td>
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<td>ALI epithelial culture</td>
<td>N = 7</td>
<td>N = 7</td>
<td>N = 7</td>
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</tbody>
</table>

CT, Computed tomography; InfTurb, inferior turbinate. |
for another 3 weeks by changing the medium at the basal compartment with DMEM:BEGM (1:1) medium. Fresh altretinoin acid (Sigma-Aldrich) was added at a concentration of 100 ng/mL, basolaterally to differentiate cells. The medium was replaced every other day. Differentiated state of the epithelial cells was evaluated by performing immunofluorescence staining of acetyl-beta-mannosidase, mucin 5AC (goblet cell marker), and E-cadherin (junctional marker) in ALI cultures at day 21 as previously presented.35 ALI-cultured epithelial cells were used for in vitro DEP exposure studies on day 21.

Preparation and exposure of DEP
We used 2 types of DEPs, which were commercially available from the National Institute of Standards and Technology (NIST, Gaithersburg, Md): diesel PM standard reference material (SRM) 1650b and SRM 2975. DEPs were weighed and dispersed in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a concentration of 50 mg DEP/mL. Dissolved DEPs were stored at −20°C until biological analysis. DEP aliquots were thawed and resuspended in an airway epithelial cell growth medium or DMEM:BEGM (1:1) medium. The suspension was sonicated for 10 minutes at 4°C with amplitude 30% and no pulse (Model VCX 130; Sonics & Materials Inc, Newton, Conn) before exposure to hNECs. Particle suspension solutions were prepared freshly. Then, the medium was treated to either submerged-cultured epithelial cells or ALI-cultured epithelial cells. For the submerged condition, we delivered 2 doses of DEPs (20 μg/mL; 2.08 μg/cm² or 50 μg/mL; 5.21 μg/cm²). For the ALI condition, we directly added 150 μL of DEP suspension to the apical side of the transmembrane (50 μg/mL; 26.3 μg/cm²), which was maintained for up to 72 hours. The medium mixed with DMSO was used as a mock control. To evaluate the effects of signaling of nuclear factor kappa B and ERK on DEP-induced ZEB2 expression, hNECs were preincubated with vehicle or inhibitor (BAY 11-7085 and PD 98059, respectively) for 1 hour, and then treated with induced ZEB2 expression, hNECs were preincubated with vehicle or inhibitor (BAY 11-7085 and PD 98059, respectively) for 1 hour followed by DEP treatment. For in vivo experiments, suspensions were prepared in PBS in a similar manner, as mentioned above, and 20 μg of DEP suspension was instilled intranasally to induce NPs in the mice nasal cavities. At each administration, 10 μL of the same suspension was applied to the nasal cavity of the mice twice a day using a NanoScope Attune nebulizer (Model 1500; Nebulizer Inc, Montreal, Canada) to aerosolize the nanobubbles at a constant rate of 0.0835 mL/min (Rnose) and diluted with compressed room air using an external pump at a flow rate of 3 L/min. Safety factor for calculation of bias (bias) was set to 2.5. The nebulizer was connected to the top part of the central tower from which the created aerosol flowed down to the peripherally settled mice. Each mouse was held in a soft restrainer such that only the nose was exposed to the aerosol cloud (Fig 7, B). Delivered dose of DEP (Ddel) was calculated following the manufacturer’s recommendations (12.5 μg/g of mice).

Murine NP model and DEP administration
Wild-type 4-week-old BALB/c (20-25g) mice were purchased from Central Laboratory Animal (Seoul, Korea) and maintained in specific pathogen-free conditions. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-160807-7-6 and SNU-191211-2-2) and complied with the governmental and international guidelines of animal experiment. The methods to induce NPs in nasal cavity were generated from allergic rhinosinusitis model, in mice with slight modifications as described previously.33,34 Briefly, the mice were sensitized and in a control group (group A: n = 10) and 4 experimental groups (group B, C, D, and E: n = 10 each). House dust mite (HDM) extract from species Der f (Gree Laboratories, Lenoir, NC) was used in this study. For sensitization, control group mice were immunized with an intraperitoneal injection of PBS (Sigma-Aldrich) plus 100 μL complete Freund’s adjuvant (Sigma-Aldrich) at day 0 and then boosted at day 5. The mice in experimental groups (B-E) were systemically sensitized by intraperitoneal injection with 100 μg of HDM extract in the presence of 100 μL complete Freund’s adjuvant. One week after the last immunization, mice were challenged by intranasal administration of 40 μL PBS (group A) or 20 μg HDM extract dissolved in 40 μL PBS (groups B-E) for 7 consecutive days. Prolonged inflammation was maintained in the experimental groups by the subsequent nasal administration with 20 μg HDM extract 3 times a week for 4 consecutive weeks. To investigate the effect of DEP, HDM-sensitized mice received either vehicle (group B) or DEP (group C, dose of 20 μg per instillation), HDM extracts (group D, dose of 20 μg per instillation), or both (group E) administered intranasally 3 times a week for 8 weeks, and were sacrificed 1 day after the last exposure (Fig 4). PBS was applied for both systemic and local stimulation in group A. To evaluate the effect of ZEB2, HDM plus DEP-treated mice were used as an NP control group. The experimental groups were challenged with lentiviral vectors (shCont-GFP or shZEB2-GFP; see Figs 6 and 7 for detailed schedule). In some experiments, mice were exposed to DEP using a nose-only exposure system as described above.

Immunofluorescence and confocal microscopy
ALI-cultured hNEC immunostainings were directly performed on transwell membranes. hNECs were incubated with the vehicle, DEP 1650b and DEP 2975 (0, 50 μg/mL) for 48 hours, respectively. Then, hNECs were fixed in 4% paraformaldehyde (Bioseasang, Seoul, Korea) for 20 minutes at room temperature, washed 3 × 10 minutes in PBS, permeabilized with 0.25% Triton X-100 in PBS (PBST) for 30 minutes, blocked in PBST containing 5% BSA (BSA-PBST) for 2 hours, incubated with primary antibodies diluted in BSA-PBST overnight at 4°C, washed 3 × 10 minutes with PBST, counterstained in secondary antibodies in BSA-PBST for 1 hour in the dark at room temperature, washed 3 × 10 minutes with PBST, stained with 4′,6-Diamidino-2-phenylindole (Sigma-Aldrich) for 10 minutes at room temperature, and mounted to glass slides using mounting medium (Dako, Carpinteria, Calif). The primary antibodies used are summarized in Table E1 in this article’s Online Repository at www.jacionline.org. For mounting on slides, membranes were cut with a razor blade and mounted. The F-actin cytoskeleton was stained with rhodamine-conjugated phallolidin (Invitrogen, Carlsbad, Calif) according to the manufacturer’s recommendations. Fluorescent images were collected on a confocal microscope (LSM 700; Carl Zeiss Microscopy, Göttingen, Germany), with ×40 objective. Scale bars were added, and images were processed using Zen Blue software (Zeiss) and photoshop CS5 (Adobe Systems Inc, San Jose, Calif).

Histology and immunohistochemistry of animal tissues
Detailed experimental protocols for processing the sinonasal tissue specimens and immunohistochemistry were described previously.33,34 Briefly, the skin on the head was removed and the mandibles were excised. The heads of the mice were fixed in 4% paraformaldehyde for 1 day, decalified in 5% nitric acid (Sigma-Aldrich) for 3 days at 4°C, paraffin embedded, and cut into 4-μm sections. Sections were stained with hematoxylin (Vector Labs, Burlingame, Calif) and eosin (Sigma-Aldrich) for evaluating poly-like lesions and epithelial disruptions, Sirius red (Polysciences Inc, Warrington, Pa) for evaluating eosinophils, Masson’s trichrome (Sigma-Aldrich) for measuring collagen depositions, and periodic acid-Schiff (Sigma-Aldrich) for counting goblet cells, according to the manufacturer’s instructions. For immunohistochemistry, sections were stained with E-cadherin, ZEB2, neutrophil elastase, and proliferating cell nuclear antigen (PCNA) antibodies. Detailed information is delineated in Table E1. Five randomly selected hpf per mouse were evaluated by 2 independent experienced examiners blinded to the groups. The criteria for NPs and epithelial disruptions were described previously.33,34 The number of NPs and epithelial disruptions were quantified microscopically from 5 coronal sections, and presented as a total number. The number of eosinophils, neutrophils, goblet cells, and PCNA-positive cells were expressed as the average numbers from 5 hpf. E-cadherin-negative spots were counted as described previously.35
Western blot analysis

Protein concentrations were measured using BCA protein assay kit (Pierce, Bonn, Germany) according to the manufacturer’s protocol. Equal amount of protein from each cell lysate was loaded on an 8% to 15% SDS-polyacrylamide gel, separated by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, Mass). The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween-20 for 1 hour at room temperature, and incubated with the appropriate primary antibodies overnight at 4°C and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 1 hour at room temperature. The antibodies used are described in Table E1. Blots were visualized using Luminata western HRP chemiluminescence substrates (Millipore), ECL Plus, or ECL Select. Immunoblotting for β-tubulin or α-tubulin served as a protein loading control. Scanned images of western blots were quantified using ImageJ software (NIH Image processing analysis, http://rsb.info.nih.gov/ij/).

Small-interfering RNAs transfection and small hairpin RNAs lentiviral vector transduction

Small-interfering RNAs (siRNAs) were purchased from IDT (Iowa, Coralville, Iowa). The nucleotide sequences (5’ to 3’) of siRNAs are presented in Table E2 in this article’s Online Repository at www.jacionline.org. For transient gene silencing, cells at 60% confluence were transfected with siRNAs using Mirus TransIT-X2 Dynamic Delivery System (Mirus Bio, Madison, Wis) according to the manufacturer’s protocol. Stealth RNAi negative control duplex (Invitrogen, Carlsbad, Calif) was used as control. To deliver the RNA interference oligonucleotides targeting ZEB2 to differentiated epithelial cells in vitro, we transfected with ZEB2-siRNA complexed with lipofectamine RNA MAX (Invitrogen) following the previous reports.36 For transduction, pGFP-C-shLenti-ZEB2 and pGFP-C-shLenti vectors (for negative controls) were obtained from OriGene (Rockville, Md). The viral vectors were cotransfected with pOG2-VSVG, pRSV-RRE, and pCMV.gRRE helper RNA (HEK293T cells), and the viral supernatant was collected. Dilutions of virus in culture medium with polybrene (Sigma-Aldrich; 5 μg/mL) were added to each well. Viral supernatants were concentrated by ultracentrifugation (20,000 RPM for 1 hour), and treated to mice nasal cavity.

Gene set enrichment analysis

Enrichment analysis was performed using the gene set enrichment analysis (GSEA) software v4.0.3, and a formatted GCT file was used as input for the GSEA algorithm (available from http://www.broadinstitute.org/gsea). The normalized data sets (GSE 107481, E-MTAB-5157, and GSE63962) used for GSEA analysis were imported from gene expression omnibus and EuroGene, maintained at 37°C. Hydrogen peroxide (H2O2; 1 mM) was added on the cultured cells for 24 hours incubation, absorbance was measured at 450 nm and 600 nm as a reference wavelength using a microplate reader (Varioskan; Thermo Electron Co, Waltham, Mass). All assays were performed in triplicate. Lactate dehydrogenase activity was calculated following the manufacturer’s instructions provided by the EZ-LDH Cytotoxicity Assay Kit (DoGEN, Seoul, Korea). Briefly, hNECs were grown in 96-well culture plates at a density of 5 × 10^4 cells per well in 200 μL of culture medium. Cells were maintained at 37°C in a 5% CO2 humidified environment for 16 hours in an airway epithelial cell growth medium (PromoCell, Heidelberg, Germany). Then, the culture medium was replaced with 200 μL of the fresh culture medium containing DMSO for mock control, or DEP (DEP1650b and DEP 2975) at concentrations of 10, 20, and 50 μg/mL. Hydrogen peroxide (H2O2; 1 mM) was added to the cultured cells for 24 hours as a positive control. At the end of incubation, absorbance was measured at 450 nm and 600 nm as a reference wavelength using a microplate reader (Varioskan; Thermo Electron Co, Waltham, Mass). The number of permutations was set to 1000.

RNA sequencing and weighted gene coexpression network analysis

Total RNA was prepared following the standard TRIZol manufacturer’s protocol (Invitrogen). Three biological replicates, treated with either vehicle or DEP, were used for RNA sequencing. RNA quality was checked by an Agilent 2100 bioanalyzer using an RNA 6000 Nano chip (Agilent Technologies, Amstelveen, the Netherlands), and RNA quantification was validated using ND-2000 spectrophotometer (ThermoFisher Scientific, Inc, Wilmington, Del). Sequentially, rRNA was eliminated from each 5-μg sample of total RNA using Ribo-Zero Magnetic kit (Illumina, Inc, San Diego, Calif). Library preparation was performed by E-Biogen (E-Biogen, Inc, Seoul, Korea) using a SMARTer Stranded RNA-Seq Kit (Clontech Lab Inc, Palo Alto, Calif) following the manufacturer’s instructions. Briefly, a strand for cDNA library is synthesized on the basis of a modified N6 primer. When the SMARTerScribe Reverse Transcriptase approaches the 5’ end of the RNA fragment, the enzyme’s terminal transferase activity adds nucleotides to the 3’ end of the cDNA. The SMARTer Stranded Oligo base pairs, with nontemplated nucleotides, create an extended template to facilitate the SMARTerScribe RT to continue replication to the end of the oligonucleotide. The cDNA, which has full-length and single-stranded, includes the complete 5’ end of the mRNA, as well as sequences that are complementary to the SMARTer Stranded Oligo. cDNA is produced to release the library, and the library is amplified. Then, we introduced barcodes to the library. High-throughput sequencing was conducted as paired-end sequencing using HiSeq 2500 (Illumina, Inc), 101-bp-long reads to minimum of 40 million reads per sample. mRNA-Seq reads were mapped using TopHat software tool to obtain the alignment file.37 Differentially expressed gene was determined on the basis of counts from unique and multiple alignments using coverage in Bedtools.38 The RT (Read Count) data were processed on the basis of Quantile normalization method using EdgeR within R (R Development Core Team, 2016) using Bioconductor.39 The alignment files were also used for assembling transcripts, estimating their abundances, and detecting differential expression of genes or isoforms using cufflinks. We used the FPKM (fragments per kilobase of exon per million fragments) as the method of determining the expression level of the gene regions. The weighted gene coexpression network analysis (WGCNA) was performed using the WGCNA package in R software.40 WGCNA determines modules using a hierarchical clustering of gene expression values. The networks were computed at the smallest power that exceeds the scale-free topology fit index higher than 0.90 (a soft power = 10). We identified a total of 37 significant modules. Then, we conducted GO annotations for human using the org.Hs.sgd.db package in R software and examined the “biological processes” category. Among the biological processes, Bonferroni-based P values less than .05 were considered significant.

Lactate dehydrogenase assay

Cytotoxicity at each time point was calculated following the manufacturer’s instructions provided by the EZ-LDH Cytotoxicity Assay Kit (DoGEN, Seoul, Korea). Briefly, hNECs were grown in 96-well culture plates at a density of 5 × 10^4 cells per well in 200 μL of culture medium. Cells were maintained at 37°C in a 5% CO2 humidified environment for 16 hours in an airway epithelial cell growth medium (PromoCell, Heidelberg, Germany). Then, the culture medium was replaced with 200 μL of the fresh culture medium containing DMSO for mock control, or DEP (DEP1650b and DEP 2975) at concentrations of 10, 20, and 50 μg/mL. Hydrogen peroxide (H2O2; 1 mM) was added to the cultured cells for 24 hours as a positive control. At the end of incubation, absorbance was measured at 450 nm and 600 nm as a reference wavelength using a microplate reader (Varioskan; Thermo Electron Co, Waltham, Mass). All assays were performed in triplicate. Lactate dehydrogenase release was calculated by the manufacturer’s recommendation.

FACS analysis of cell death

Effect of DEP on cell death such as apoptosis and necrosis was determined by the combined application of Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (BD Pharmingen, Heidelberg, Germany) and propidium iodide (Sigma-Aldrich), followed by flow cytometric analysis according to the manufacturer’s protocol. Briefly, vehicle- or DEP-treated cells at each time points were washed with PBS and removed from the plates by trypsinization. The cells were resuspended in a binding buffer, and then stained with FITC-conjugated annexin V antibody for 15 minutes in the dark at room temperature. Sequentially, cells were treated with propidium iodide. Hydrogen peroxide (H2O2; 1 mM) was added on the cultured cells for 24 hours for positive controls. Flow cytometric analysis was immediately performed in a BD FACS Canto Flow Cytometer (BD Biosciences, San Jose, Calif).
Measurement of transepithelial electrical resistance and permeability of dextran

hNECs (1 × 10^5 cells per well) were seeded onto transwell 0.4-μm-pore-size filters (Costar, Corning, Inc) in 24-well plates. After confluence, monolayer cells were exposed to ALI conditions, and induced differentiation by removing the medium in the apical compartment as described previously.32 Transepithelial electrical resistance (TEER) measurements were performed at 0, 24, 48, and 72 hours after exposure to either vehicle or DEP (50 μg/mL; 26.3 μg/cm^2), epithelial integrity was determined by using an EVOM/Endohm chamber (WPI, Sarasota, Fla), and corrected by subtracting the background to the blank transwell inserts and medium. For TEER measurements, 0.2 and 1.0 mL of preequilibrated medium was added to the apical and basolateral reservoirs, respectively. Wells not building up sufficiently (TEER > 3000 Ω × cm^2) were not included in experiments. Paracellular flux measurements were conducted after TEER experiments. Paracellular permeability was measured at 72 hours after apical addition of FITC-dextran (2 mg/mL) (Sigma-Aldrich) for 3 hours based on the intensity of FITC in the basal compartment medium (100 μM), as measured with an ELISA reader at 480 nm (Varioskan; Thermo Electron Co). The values of TEER and permeability in ALI-cultured hNECs transfected with ZEB2 siRNA complexed with RNAiMAX following the previous reports36 were evaluated similarly as described above. Each experiment was performed in triplicate.

RNA extraction and real-time quantitative PCR

Total RNA was isolated using QIAshredder and RNeasy Plus Mini Kit (both Qiagen, Valencia, Calif) following the manufacturer’s instructions. cDNA synthesis was carried out from 1 μg of RNA using Tetro cDNA-synthesis kit (Bioline, London, UK) following the manufacturer’s recommendations. PCR was done with SYBR Green Polymerase Chain Reaction Master Mix as a ready-to-use reaction mixture (Enzymics, Daejeon, Korea) in 96-well optical plates using the CFX Connect Real-Time Polymerase Chain Reaction Detection System (Bio-Rad Laboratories, Inc, Hercules, Calif). Cycling conditions were 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds, 60°C for 1 minute, and 72°C for 30 seconds. All genes were normalized to reference genes (GAPD for human genes and ACTB for mouse genes), and the results are presented as relative fold changes using the comparative 2^ΔΔCt method. cDNA was amplified by using the primers described in Table E3 in this article’s Online Repository at www.jacionline.org.

RESULTS

DEP treatment promotes EMT and cell migration potential in AECs

We first applied the GSEA to hallmark gene sets and GO gene sets to decipher the cellular consequences of DEP exposure. In this process, we used 3 publicly available gene expression databases (GSE 107481, E-MTAB-5157, and GSE 63962). We observed several gene sets that were enriched in the DEP-treated group (293, 880, and 523 gene sets for GSE 107481, E-MTAB-5157, and GSE 63962, respectively) compared with the vehicle-treated group (P < .05; false discovery rate [FDR] < .30). We then searched for the gene sets commonly upregulated in the 3 different data sets. The EMT gene set was ranked number 1 among the subset of gene sets (Fig 1, A and B). Representative enrichment plots are shown in Fig 1, C. To compare the cellular consequences of DEP administration on the upper and lower AECs, we treated ALI-cultured hNECs (upper AECs) with DEP following the previous reports,32 and conducted RNA sequencing (Fig 1, D). A study has reported that the transcriptome has a reproducible coexpression configuration that offers a framework for speculating disease biology.33 Therefore, we performed WGCNA to investigate modules of genes that change with similar patterns following DEP exposure in ALI-cultured hNECs.42 WGCNA identified 37 gene modules correlated with DEP exposure (Fig 1, E). Color label identifies represent each module, and each color shows a cluster of genes with a similar expression pattern across samples. Turquoise and blue modules showed the most intense association (modSize > 1000 and Bonferroni-corrected P values < .05). After GO analysis using the genes included in turquoise and blue modules, we ranked the enriched GO terms on the basis of biological process following the Bonferroni-corrected P values. Among the 14 GO terms, 7 GO terms (50%) were related to cytokoskeleton remodeling and cell migration (Fig 1, F). Collectively, these results support the speculation that DEP exposure could induce EMT in hNECs.

DEP exposure induces EMT in hNECs

Considering that EMT and cytokoskeleton remodeling are the prerequisite processes for cell migration,43,44 we examined whether DEP exposure was responsible for the EMT in hNECs. hNECs were exposed to SRM DEP 1650b and SRM DEP 2975 in a concentration-dependent manner. In these concentrations, we could not observe the effects of DEP in terms of cell toxicity, apoptosis, and necrosis as confirmed by lactate dehydrogenase assay, Annexin V/propidium iodide assay, or additional immunoblot assay, respectively (see Fig E1, A–C, in this article’s Online Repository at www.jacionline.org). Although hNECs cultured with vehicle possess an epithelial morphology, hNECs exposed to DEP showed a larger, more spindle/fibroblasts-like shape (Fig 2, A, left). Consistently, hNECs developed lamellipodia after DEP treatment (Fig 2, A, right). To assess the EMT phenomenon under physiological conditions, we delivered DEP to ALI-cultured hNECs. DEP exposure reduced the E-cadherin expression (Fig 2, B). In addition, DEP increased the protein levels of mesenchymal markers (N-cadherin and α-SMA), but decreased those of epithelial markers (E-cadherin and ZO-1) (Fig 2, C and D). Barrier dysfunction is strongly associated with the progression of EMT and features of CRC.45,46 We therefore checked whether the barrier function of epithelial cells could be impaired...
FIG 1. DEPs increase EMT and cell migratory potential in AECs. A and B, GSEA. Venn diagrams show 17 commonly upregulated gene sets ($P < .05$ and FDR < 0.30) by DEP treatment. Among them, top 10 gene sets are listed. C, Enrichment plots for top 1 ranked gene set of the GSEA in the data set of GSE 107481, E-MTAB-5157, and GSE 63962. D, Schematic diagram of DEP exposure and RNA sequencing in ALI-cultured hNECs ($n = 6$). E, WGCNA dendrogram, which detects genes with similar expression patterns and classifies them into modules (represented by colors) (scale-free topology fit index $> 0.90$, a soft power $= 10$) ($n = 6$). F, Enriched GO terms in the turquoise and blue modules were ranked by the Bonferroni $P$ values. GO terms based on BP with modSize more than 1000 and Bonferroni-corrected $P$ values less than .05 are shown. Similar BP terms are highlighted in red colors. BP, Biological process.
**FIG 2.** DEPs induce EMT in hNECs. A, Representative phase contrast images and F-actin structures of hNECs. Scale bars = 200 μm (×100, left) and 50 μm (×400, right). Arrows indicate lamellipodia. Scale bar = 20 μm. B, Representative immunofluorescence images of ALI-cultured hNECs exposed to DEP. E-cadherin (green) and DAPI (blue). Scale bar = 20 μm. C and D, Representative immunoblots of EMT markers in hNECs after DEP exposure. E, TEER was measured after DEP exposure in ALI-cultured hNECs. F, Paracellular flux in response to DEP exposure in ALI-cultured hNECs. G, mRNA expression of EMT-regulating transcription factors was assessed in ALI-cultured hNECs (n = 6). H, hNECs cultured with DEPs were examined for the expression of EMT-regulating transcription factors. Black dashed line (---) indicates vehicle-treated cells normalized to 1-fold. Heat maps were generated from normalized data. I, Representative immunoblots and quantification of ZEB2 protein expression in hNECs (n = 3). In all immunoblot analyses, protein intensities were quantified by ImageJ software, normalized to β-tubulin. All results are presented as means ± SEM. Individual values are indicated by dots (Fig 2, F, G, and I). Statistical significance was determined by Student t test (Fig 2, H and I) and Wilcoxon matched-pairs test (Fig 2, E and F). DAPI, 4′,6-Diamidino-2-phenylindole. **P < .01.
by DEP treatment. As surrogate markers of barrier function in ALI-cultured hNECs, TEER and paracellular diffusion of FITC-dextran assays were used. DEP reduced the TEER levels until 72 hours (Fig 2, E), whereas the permeability of FITC-dextran through DEP-treated epithelial cells was higher than that of the vehicle-treated cells (Fig 2, F). These data indicate that exposure to DEP disrupts epithelial barrier and promotes EMT in hNECs. Next, we focused on previously known EMT-regulating transcription factors to investigate mechanisms through which EMT occurs. We noted that normal hNECs isolated from 6 different control subjects possess similar mRNA expression levels of EMT-regulating genes except SLUG, ZEB1, and TWIST2 (Fig 2, G). To determine the factors that were commonly increased by DEP exposure, we treated hNECs with either DEP 1650b or DEP 2975 and assessed their mRNA expressions (Fig 2, H). Although both ZEB2 and SLUG mRNA levels were significantly increased by the DEP treatment (Fig 2, H), we could confirm only ZEB2 induction at the protein level in hNECs (Fig 2, I). We then investigated which molecular signaling pathways are attributed to ZEB2 upregulation in response to DEP treatment. Among the key signaling pathways, we observed significant activation of nuclear factor kappa B and ERK by DEP exposure (see Fig E2, A, in this article’s Online Repository at www.jacionline.org). However, only nuclear factor kappa B inhibition consistently reduced DEP-induced ZEB2 expression (Fig E2, B). Considering that DEP can induce oxidative stress in cells, we also tested the effect of N-acetyl-L-cysteine on DEP-treated cells. As expected, N-acetyl-L-cysteine treatment slightly reduced the DEP-induced ZEB2 levels (Fig E2, C). We further determined whether DEP-induced ZEB2 was attributable to DEP extracts or particles themselves. Thus, we treated hNECs with polystyrene-based microparticles (0.1, 0.5, and 1.0 μm) and compared ZEB2 expression. However, microparticles induced modest ZEB2 expression as compared with DEP (see Fig E3, A, in this article’s Online Repository at www.jacionline.org). When hNECs were incubated with 2 types of non-PM2.5 fine dust (with aerodynamic diameter <10 μm [PM10]-like), which contain polycyclic aromatic hydrocarbons or trace elements, as well as HDM, as an environmentally exposable allergen, the PM10 fine dust induced ZEB2 expression as much as DEP did (Fig E3, B). Together, the results suggest a contribution of ZEB2 to DEP-induced EMT, and the increase could result from the DEP extracts rather than from the particles themselves.
FIG 4. DEPs trigger NPs and epithelial remodeling in HDM-sensitized AR murine model. A, Schematic illustration of the murine NP model. B, Representative hematoxylin and eosin–stained images of sinonasal spaces and polypoid lesions from indicated groups (scale bar = 200 μm). Asterisk denotes the NP lesions. Areas indicated with squares are shown as magnified images (scale bar = 50 μm). C and D, The numbers of NPs and epithelial disruptions. E, The numbers of E-cadherin (−) spots per hpfs were counted and compared. F, Subepithelial collagen thickness. G, The ratio of nuclear positive PCNA. H, ZEB2 mRNA levels. I and J, CDH1 and ACTA2 mRNA levels. All results are presented as means ± SEM (n = 5, respectively). Individual values are indicated by dots (Fig 4, C–J). Statistical significance was determined by Kruskal–Wallis tests (P < .01), followed by Mann-Whitney U test for pairwise comparisons (Fig 4, C–J). *P < .05; **P < .01.
FIG 5. DEP exposure aggravates EMT characteristics in epithelial cells derived from patients with CRS ex vivo. Representative immunoblots (A and B) and quantification (C and D) of E-cadherin and ZEB2 expressions in ALI-cultured hNECs from healthy control subjects (n = 4), patients with CRSsNP (n = 7), and patients with CRSwNP (n = 7), exposed for 72 hours or unexposed to DEPs (DEP 1650b or DEP 2975) at concentration of 50 μg/mL. In all immunoblot analysis, protein intensities were quantified by ImageJ software, normalized to β-tubulin and pooled control. E, Correlation of ZEB2 fold change and E-cadherin fold change in each group of subjects. Statistical significance was determined by Wilcoxon matched-pairs test (Fig 5, C and D) and Spearman correlation test (Fig 5, E). Spearman r values and the corresponding P values were calculated. *P < .05; **P < .01.
DEP-induced EMT is dependent on ZEB2 expression

To directly address this hypothesis, we used an siRNA to target ZEB2 followed by DEP treatment. As shown in Fig 3, A, and Fig E4 (in this article’s Online Repository at www.jacionline.org), ZEB2 knockdown restored DEP-induced EMT as evidenced by an increase in epithelial markers (E-cadherin and ZO-1) and a decrease in mesenchymal markers (N-cadherin and α-SMA). Consistently, knockdown of ZEB2 reduced the numbers of DEP-induced F-actin stained with phalloidin (Fig 3, B). We also performed TEER analysis and measured the amount of paracellular flux after DEP exposure in a time-dependent manner until 72 hours. Consistently, TEER decreased by DEP administration after 48 hours of exposure. In addition, paracellular flux markedly increased in ALI-cultured hNECs after 72 hours of exposure to DEP, confirming the previous results. However, knockdown of ZEB2 followed by DEP exposure restored TEER values and decreased the levels of paracellular flux (Fig 3, C and D). These results reveal that DEP exposure induced EMT via ZEB2 expression in hNECs.

Effects of DEP on polyp formation in a murine NP model

Next, we would like to investigate the potential pathophysiological relevance of ZEB2-induced EMT with regard to respiratory airway diseases. EMT is a feature of CRS disease and has been implicated as a possible mechanism in the pathogenesis of CRSsNP. We previously reported that EMT contributes to the formation of NPs.32,34,47 Therefore, we tested whether DEP exposure could induce NPs. To determine the effect of DEP on NP formation, we used an experimental NP model based on previous reports with some modifications.33,34,48 BALB/c mice were sensitized intraperitoneally with either PBS or HDM twice. After prolonged inflammation by HDM administration, mice were exposed intranasally to PBS (vehicle), DEP, HDM, and HDM plus DEP 3 times a week over an 8-week period (Fig 4, A). Interestingly, we found a synergistic increase in NP numbers and the number of epithelial disruptions in mice exposed to HDM plus DEP compared with that observed in either DEP- or HDM-exposed mice. DEP exposure alone did not promote NP formation (Fig 4, B-D). Fig E5 in this article’s Online Repository at www.jacionline.org shows the representative photographs of NPs counted in Fig 4, C. Next, we checked whether DEP-induced EMT weakened epithelial integrity and promoted epithelial remodeling in vivo by analyzing E-cadherin expression, collagen amount, cell proliferation rate, and goblet cell hyperplasia. We counted the E-cadherin–negative spots as mentioned in previous reports,33,35,36 and the results are shown in Fig 4, E. Exposure to either DEP or HDM sufficiently reduced the levels of E-cadherin. This effect was more prominent in HDM plus DEP–delivered group (Fig 4, E). In contrast, subepithelial collagen thickness was detected the most in HDM plus DEP–administered mice (Fig 4, F). Treatment with HDM plus DEP synergistically increased the percentage of PCNA-positive cells as well as enhanced the ZEB2 mRNA levels (Fig 4, G and H). Representative images are shown in Fig E6 in this article’s Online Repository at www.jacionline.org. In addition to HDM treatment, DEP exposure further attenuated the mRNA levels of E-cadherin (epithelial marker), but upregulated α-SMA (mesenchymal marker) in mice nasal mucosa tissues (Fig 4, I and J). These results from an in vivo system are in agreement with those of the in vitro experiments, confirming the effects of DEP on hNECs.

Coexposure to HDM and DEP had minor effects on inflammatory response in vivo

Previous studies used various kinds of immunization protocols and addressed an adjuvant effect of DEP that enhanced Th2-related cytokines and IgE production.17,49 We therefore examined whether DEP affected inflammatory cytokine secretion or immune cell infiltration. HDM/DEP exposure did not further increase the number of eosinophils and Th2-related cytokines, such as IL-4 and IL-13, which were induced by HDM treatment. Similarly, repeated DEP exposure over an 8-week period did not result in a marked increase in total IgE plasma levels compared with those observed in the vehicle-administered mice. Exposure to both HDM and DEP also did not enhance total IgE levels compared with those treated with HDM alone However, the numbers of neutrophils and goblet cells were slightly upregulated following HDM/DEP treatment (see Fig E7, A-F, in this article’s Online Repository at www.jacionline.org). Furthermore, the mRNA levels of CXCL1 and CXCL2, but not CXCL5, increased in the HDM + HDM/DEP–treated group when compared with those in the HDM + HDM–treated mice (see Fig E8 in this article’s Online Repository at www.jacionline.org). This result suggests that coexposure to HDM and DEP had modest effects on allergic inflammation. Collectively, epithelial remodeling might be causing NP formation in mouse nasal cavity rather than the inflammatory adjuvant effect of DEP.

DEP exposure exacerbates EMT in CRS patient–derived epithelial cells and shows negative correlation between E-cadherin and ZEB2 ex vivo

Our data showed that DEP promoted EMT in hNECs, whereas DEP-exposed mice did not exhibit significant induction of NP formation (P = .136). However, coexposure of HDM and DEP induced NPs in mice. These discrepant results led us to hypothesize that during CRS progression, primed epithelial cells respond more strongly to extrinsic cues such as DEP than the normal epithelial cells and resulted in NP formation. A previous report showing that human respiratory epithelial cells have allergic inflammatory memory also supports our hypothesis.37 To test this hypothesis, we seeded hNECs from control subjects, patients with CRSsNP, and patients with CRSsNP into ALI cultures for 3 weeks ex vivo. After treating ALI-cultured epithelial cells with DEP for 72 hours, we performed immunoblot assay and compared the levels of E-cadherin and ZEB2. First, we observed that the baseline ZEB2 levels were already increased in the cells in patients with CRS and upregulated more in patients with NP. However, the expression of E-cadherin exhibited opposite trends. Considering CRSsNP revealed more severe forms of EMT characteristics compared with CRSsNP and normal tissues, upregulation of endogenous ZEB2 levels in NP-derived epithelial cells is reasonable. Western blot analysis revealed that ZEB2 expression was markedly induced by DEP treatment, but E-cadherin levels decreased mostly in CRS patient–derived epithelial cells (Fig 5, A-D). However, DEP exposure to the cells derived from either normal subjects or patients with NPs presented mild changes in terms of ZEB2 and E-cadherin levels. The fold change of ZEB2 expression was negatively correlated with that of E-cadherin expression.
FIG 6. Tissue-specific knockdown of ZEB2 diminishes HDM/DEP-induced nasal polypoid lesions. A and B, LA4 cells transduced with either shCont-GFP or shZEB2-GFP for 8 hours in polybrene (5 μg/mL)-containing medium were analyzed using FACS (left) and quantitative RT-PCR (right) (n = 3). C, Protocol for the murine NP model. BALB/c mice were treated with HDM, DEP, and shCont-GFP or shZEB2-GFP lentiviral vectors. i.n., Intranasal; i.p., intraperitoneal. D, Photographs of representative nasal polypoid lesions stained with hematoxylin and eosin in the indicated groups (scale bar = 200 μm). Asterisk denotes NP lesions. Areas indicated
expression (Fig 5, E). These results indicate that defective epithelial cells such as CRS patient–derived epithelial cells have a greater tendency to induce EMT.

**Tissue-specific ZEB2 knockdown reduces HDM/DEP-induced NPs in mice**

To verify the role of ZEB2 in the process of NP formation, we manipulated ZEB2 in a tissue-specific manner by delivering lentiviral vectors to an HMD/DEP-induced NP murine model. We first tested the transduction efficiency of small hairpin RNA targeting ZEB2 in an LA4 cell line, and found that LA4 cells transduced with lentiviral-mediated ZEB2-specific small hairpin RNAs inhibited ZEB2 expression (Fig 6, A and B). We then delivered ZEB2 small hairpin RNAs to the HDM/DEP-induced NP murine model (Fig 6, C). As expected, HDM/DEP-treated mice showed a greater number of NPs, but the intranasal administration of shZEB2 lentiviral vectors reduced mucosal inflammation with multiple polypoid lesions (Fig 6, D and E). Immunohistochemical analysis showed the changes in ZEB2 promoted by DEP on nasal tissues (Fig 6, F). E-cadherin expression was also restored by the nasal mucosa–specific knockdown of ZEB2 in HDM/DEP-treated mice (Fig 6, G and H). In addition, the number of PCNA-positive cells decreased following the shZEB2 delivery (see Fig E9, A, in this article’s Online Repository at www.jacionline.org). These results reveal that ZEB2 is required for the EMT process in HDM + DEP–induced NP formation in mice.

**Tissue-specific ZEB2 inhibition decreases nasal polypoid lesions in a nose-only DEP exposure system**

We next investigated whether ZEB2 induction occurred in an in vivo system using a nose-only exposure system closer to physiological conditions. Considering that ZEB2 is a transcription factor, we checked ZEB2 expression at early time points (day 1 and day 5) after DEP exposure (Fig 7, A). BALB/c mice were strained inside nose-only exposure tubes and exposed to either vehicle or DEP for 1 hour a day, resulting in the specific delivery of DEP to nasal cavity (Fig 7, B). Notably, ZEB2 mRNA levels increased even after 1-hour exposure to DEP, and were further upregulated in the 5 days of DEP exposure (Fig 7, C). Finally, we also validated the effects of ZEB2 inhibition by exploiting a nose-only inhalation system (Fig 7, D). Similarly, intranasal delivery of shZEB2 lentiviral vectors decreased the number of NPs (Fig 7, E and F), diminished ZEB2 expression levels (Fig 7, G), but restored E-cadherin levels (Fig 7, H and I). In terms of PCNA numbers, similar findings were observed, as shown in Fig E8, A (Fig E9, B). Overall, these results indicate that both intranasal delivery of DEP and DEP exposure using a nose-only system enhances NP formation in an HDM-sensitized AR murine model, and that ZEB2 contributes to DEP-mediated nasal polypogenesis.

**DISCUSSION**

The present study suggests that exposure to DEP induces EMT in hNECs and increases the number of NPs in mice (Figs 2 and 4). Furthermore, DEP delivery to CRS patient–derived epithelial cells triggers potent EMT features as compared with those from control subjects (Fig 5). We found that DEP-mediated EMT was attributed to ZEB2, a master regulator of the EMT, among other key EMT-regulating transcription factors such as SNAI and TWIST (Figs 2 and 3). Knockdown of ZEB2 sufficiently restored DEP-induced EMT in hNECs (Fig 3). ZEB2 blockade in mice prevented HDM + DEP–induced NP formation (Figs 6 and 7). The effects of DEP have been implicated in respiratory allergic diseases such as AR and allergic asthma; however, the mechanisms of DEP-induced upper airway diseases have not been studied. Our data provide the first evidence that DEP-induced ZEB2 contributes to NP formation by promoting EMT in hNECs. In addition, we observed that epithelial cells derived from patients with CRS might have primed memory and might have experienced extensive epithelial remodeling toward NP formation owing to DEP treatment (Fig 5). Collectively, approaches targeting inhibition of ZEB2 can be potential therapeutic strategies for patients with NPs who reside in high DEP–exposed areas.

EMT is a reversible process and is a marked characteristic of CRS with or without NPs. We previously demonstrated several times that inhibition of EMT progression based on their mechanism sufficiently blocked NP formation. In this study, we suggest that DEP exposure promotes EMT as evidenced by the WGCNA in ALI-cultured hNECs and various kinds of in vitro experiments (Figs 1-5). Despite the lack of relevant studies, a few reports support our observations. Recently, Rynning et al demonstrated that DEP-treated HBECs showed increase in EMT-related genes, but reduced migration potential as compared with the vehicle-treated HBECs. Some studies also reported that DEP exposure to HBECs could induce the expression of EMT-related genes. In addition, human lung epithelial cells (Beas-2B) treated with PM2.5 derived from Yangtze river delta region in China enhanced invasion ability and induced the expression of EMT markers. However, these studies did not show the clear EMT phenotype, related mechanisms, and also did not suggest the clinical implications of DEP exposure. Our study elucidated the specific mechanism regarding the effect of DEP on the upper AEC and examined this mechanism in the possible upper airway diseases. We exposed cells to DEPs in vitro, in vivo, and ex vivo, and confirmed the contribution of ZEB2-induced EMT in terms of nasal polypogenesis.

The carcinogenic effects of DEP are controversial, underscoring the need for further studies comparing the effects of DEP extracts and intact particles on hNECs separately. Because DEPs comprise fine particles (mainly 0.1-2.5 μm), we treated the cells with polystyrene-based microparticles (0.1, 0.5, and 1 μm) in a
FIG 7. Tissue-specific inhibition of ZEB2 alleviates HDM/DEP-induced NPs in a nose-only exposure system.
A, Schematic illustration of DEP delivery to mice using a nose-only exposure system. B, Representative photographs of mice strained inside a nose-only exposure tube (left). Mice were exposed to DEPs (bottom). Aggregation of DEPs was identified in the mouse nasal cavity (right). Arrow indicates DEP. C, ZEB2 mRNA expressions from the wild-type control mice and DEP-challenged mice (n = 6).
D, Protocol for the murine NP model. E, Photographs of representative nasal polypoid lesions stained with hematoxylin and eosin.
concentration-dependent manner, and compared them with DEP-treated hNECs. However, we hardly observed an increase in ZEB2 expression (Fig E3, A). In addition, we could not detect any morphological changes in microparticle-treated hNECs (data not shown). These results suggest that DEP-induced EMT might be affected by the DEP extracts rather than the microparticles. However, our present study has certain limitations. Polystyrene-based microparticles may somewhat differ from microparticles presented in DEP. In this regard, various types of microparticles, which could mimic microparticles in DEP, need to be tested in the future. Another limitation is that our study did not compare the effects of DEP and other air pollutants in terms of EMT in hNECs. Considering that respiratory allergic diseases are a result of air pollution, which is also caused by other pollutants such as Asian sand dust and exhaust particles, future studies will be required.

To evaluate the physiological relevance of DEP, we replicated the real-life exposure conditions as closely as possible. A number of studies have examined PM2.5 concentrations in several urbanized countries, and reported that the annual means of PM2.5 were 10 to 30 μg/m^3 (depending on the countries). Especially, exposure to DEP in occupational settings can be more extensive. Therefore, the concentrations used in our study (20-50 μg/mL for in vitro studies and 10 μg/20 μL for in vivo studies) are biologically relevant. These concentrations have also been frequently used in other studies.

The relationship between animal model systems and target pathophysiology is crucial for a comprehensive understanding of many human diseases. Intraperitoneal injection, since 1980s, has been the most traditional route of inducing sensitization. However, one of the major criticisms of animal models for airway diseases, including asthma and CRS with or without NP, is that they do not mimic the real routes when triggering allergic responses. Therefore, the use of airways for sensitization, such as via intranasal or intratracheal administration, has recently been applied to mimic human airway diseases, instead of intraperitoneal or subcutaneous routes. Multiple studies have reported delivering DEP with HDM or ovalbumin via the intranasal route for sensitization, and they observed adjuvant activity of DEP on IgE and IgG1 antibody productions. It has also been reported that inhaled HDM challenge induces a classical Th2 inflammatory mediator profile in the airway (like ovalbumin). On the basis of such results, we speculate that HDM + DEP sensitization through the intranasal route followed by HDM treatment could trigger rhinosinusitis characteristics in mice. Furthermore, it could facilitate NP formation when mice are exposed to HDM or DEP over a long period.

Notably, DEP-exposed hNECs exhibited EMT phenotypes, whereas DEP or HDM alone did not induce NPs significantly in mice (Figs 2 and 4). However, coexposure to HDM and DEP could induce NP formation. Our hypothesis is that either DEP or HDM exposure impairs epithelial cells and potentially promotes EMT in vivo; however, the threshold required to generate NPs consistently cannot be achieved. Nevertheless, the delivery of HDM + DEP mixture after HDM sensitization might damage the cells in an additive and/or synergistic manner, in turn allowing NP formation in mice. From these observations, we hypothesized that defective epithelial cells from patients with CRS possibly respond more strongly to air pollutants such as DEP rather than normal epithelial cells. To resolve this, we collected epithelial cells from normal subjects, patients with CRS, and patients with CRSwNP. Cells were cultured under ALI conditions followed by DEP treatment, and the levels of ZEB2 and E-cadherin were evaluated. The epithelial cells derived from patients with CRS showed significantly higher ZEB2 expression levels than those from normal subjects and patients with CRSwNP. We believe that CRS-derived hNECs were already primed cells, which made the cells more vulnerable to potential environmental cues, such as DEP, when compared with normal cells. Considering NP-derived epithelial cells already exhibited distinct mesenchymal features, our observation that additive DEP treatment barely affected ZEB2 and E-cadherin levels is quite reasonable.

Indeed, the association between ZEB2 expression and poor prognosis in patients with colorectal cancer has been reported several times. Another study also supports the importance of ZEB2 with respect to colon tumor metastasis. Considering gastrointestinal (GI) polyps, including colonic tumors, are another type of polyps, as observed in the nasal mucosa, exploration of the role of ZEB2 in the GI tract is valuable. DEP can be delivered to the intestinal tract via the esophagus and might affect GI epithelial cells. Consequently, DEP inhalation could induce ZEB2 expression in GI epithelial cells. However, DEP itself may not trigger intestinal polyps, as observed in the nasal spaces (Fig 4). Because the azyoxymethane/dextran sodium sulfate (AOM/DSS) murine model showed activation of multiple key signaling pathways such as Wnt/b-catenin signaling, which is a critical pathway in GI tumorigenesis including polyposis, DEP delivery based on an AOM/DSS model should be explored for use in the evaluation of GI polyps in the future.

Although our data clearly showed that a traffic-related air pollutant (DEP) promotes EMT, especially in CRS-driven epithelial cells, implying that DEP might trigger NP formation among patients with CRS, there is little epidemiological data to support our conclusion. Nevertheless, this is the first report, to our knowledge, exploring the role of DEP in nasal polypogenesis. Despite the lack of related studies, a few related reports support our findings. For example, for each unit increase in PM2.5 exposure, there was a 1.89-fold upregulation in the proportion of patients with CRSsNP who required further surgery, indicating that air pollutants are potential environmental risk factors for CRS disease exacerbation. In addition, PM10 particles were
associated with increased risk of CRS based on the Korea National Health and Nutrition Examination Survey data.\textsuperscript{71} PM\textsubscript{2.5} (10 mg/m\textsuperscript{3}) exposure is associated with increased prevalence of AR in Chinese children and worsened Rhinoconjunctivitis Quality of Life in Peruvian children.\textsuperscript{2,72} Furthermore, numerous recent studies have demonstrated a positive correlation between the frequency of AR episodes and traffic-related air pollution.\textsuperscript{74-76} However, in the studies, the levels of exposure to DEP or PM between patients with CRSsNP and patients with CRSwNP have not been studied. Therefore, well-controlled prospective studies are required in future.

**Conclusions**

We found that ZEB2-mediated EMT has a crucial role in DEP-induced nasal polypogenesis, and that ZEB2 loss apparently mitigates NP formation as well as sinonasal mucosa inflammation (Figs 4-7). Therefore, controlling ZEB2-induced EMT may be an effective target for treating nasal polypogenesis in mucosal epithelium. Furthermore, modulating ZEB2 may have a therapeutic advantage for the treatment of upper airway diseases caused by particulate-related air pollution such as that caused by DEP.

**Clinical implications:** The results of the present work highlight ZEB2 as a potential molecular target for the expansion of the options available for DEP-induced airway disease therapy, including NPs.

**REFERENCES**