

High-mobility group box 1 protein induces epithelial-mesenchymal transition in upper airway epithelial cells*

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Abstract

Background: In the treatment of rhinosinusitis, nasal polyps are a major problem, and the epithelial-to-mesenchymal transition (EMT) process is considered pivotal in their development. Although various studies have addressed the role of high mobility group box 1 (HMGB1) nuclear protein in this setting, its impact on EMT has yet to be evaluated. Our aim was the pathogenic mechanism of HMGB1 in EMT and EMT-induced upper respiratory nasal polyps.

Methods: We investigated the EMT-related effects of HMGB1 in human nasal epithelial (HNE) cells using western blot analysis, transepithelial-electrical resistance (TEER) testing, wound healing assay, and immunofluorescence. HNE cells were incubated in a low-oxygen environment to evaluate the role of HMGB1 in hypoxia-induced EMT. Further support for our in vitro findings was obtained through murine models. Human nasal polyps and nasal lavage fluid samples were collected for western blotting, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA).

Results: HMGB1 increased mesenchymal markers and decreased epithelial markers in HNE cells. Hypoxia-induced HMGB1 in turn induced EMT, apparently through RAGE signaling. We verified HMGB1-induced EMT in the upper respiratory epithelium of mice by instilling intranasal HMGB1. In testing of human nasal polyps, HMGB1 and mesenchymal markers were heightened, whereas epithelial markers were reduced, compared with tissue controls.

Conclusion: HMGB1 secretion in nasal epithelium may be a major pathogenic factor in upper respiratory EMT, contributing to nasal polyps.

Key words: HMGB1 protein, epithelial-mesenchymal transition, chronic rhinosinusitis, nasal polyps

Introduction

Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells are completely transformed, acquiring mesenchymal cellular functions and characteristics ⁽¹⁾. In doing so, profound changes occur in epithelial transcriptional regulation, cytoskeletal features and motility, cell adhesion, and extracellular matrix (ECM) synthesis ⁽¹⁾. Previous studies have found that various cytokines, chemokines, and growth factors are associated with EMT ⁽²⁾.

High mobility group box 1 (HMGB1) is a nuclear protein that

functions as a DNA chaperon. HMGB1 may be released into the extracellular space through active means (following pro-inflammatory stimulation) or on a passive basis (via leaky necrotic cellular membranes) ⁽³⁾. However, the functions of HMGB1 may vary by location, serving as molecular chaperon in the cytoplasm or as part of the damage-associated molecular pattern (DAMP) displayed in extracellular spaces ^(4,5). Recently, extracellular HMGB1 has also been recognized as an EMT enhancer in several organs. In hypopharyngeal cells, HMGB1 is essential for maintaining the interstitial-cell phenotype in TGF- β 1-induced EMT, and the

silencing of HMGB1 greatly curtails both invasive and metastatic capabilities of malignancies ⁽⁶⁾. HMGB1-induced fibroblast-to-myofibroblast differentiation in the lung is triggered by NF- κ B-mediated TGF- β 1 release, and HMGB1 liberation in injured lungs promotes EMT ^(7,8).

EMT figures prominently in the upper airways of patients with chronic rhinosinusitis (CRS), predisposing to nasal polyp formation ^(9,10). Human nasal polyps exhibit increases in pro-protein convertases (PCs), surpassing levels in normal nasal mucosa by one-third and accounting for an association with EMT ⁽¹¹⁾. Hypoxia induces EMT, and EMT contributes to nasal polyps in CRS patients ⁽¹²⁾. We have previously shown that HMGB1 correlates with the severity of inflammation in patients with CRS, and hypoxia is known to induce the secretion of HMGB1 in upper airway epithelial cells ^(4,13). However, the relation between HMGB1 and upper-airway EMT has yet to be fully investigated.

To gain functional insights, we evaluated the role of HMGB1 in EMT using primary human nasal epithelial (HNE) cells, tissue from nasal polyps, polyp-derived lavage fluids, and mouse nasal mucosa. Based on our in vitro and in vivo experimental findings, it is apparent that HMGB1 can induce an EMT-like process in the upper airway epithelium.

Methods

Reagents

Antibodies against vimentin (sc6260), α -SMA (sc53015), GAPDH (sc47724), E-cadherin (sc7870), RAGE (sc365154), and TLR4 (sc10741) (Santa Cruz Biotechnology, Dallas, TX, USA); against occludin (33-1500) and ZO-1 (40-2300) (Invitrogen [Thermo Fisher], Carlsbad, CA, USA); and against TLR2 (ab24192) (Abcam, Cambridge, UK) were all purchased, as well as enzyme-linked immunosorbent assay (ELISA) kits for human TGF- β (R&D Systems Inc, Minneapolis, MN, USA) and human HMGB1 (Shino-Test Corp, Kanagawa, Japan). RIPA lysis buffer and a BCA protein assay kit were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Acn enhanced chemiluminescent (ECL) immunodetection system (Amersham, Little Chalfont, Buckinghamshire, UK) was also purchased, and recombinant wild-type HMGB1 protein was the product of CHO cells (A&R Therapeutics, Daejeon, Republic of Korea).

Cell culture and treatment

We collected a small portion of inferior turbinate mucosa from patients during nasal surgery ⁽¹⁴⁾. Informed consent was obtained from all patients, and this study was approved by the Institutional Review Board of Yonsei University College of Medicine. Primary human nasal epithelial (HNE) cells were cultured as described previously. In our previous study, it was proved that these cells are differentiated into respiratory airway epithelial cells following our protocol, having morphological, physiological, and biochemical characteristics similar to those in

in vivo respiratory airway epithelial (Supplementary Figure 1)⁽⁴⁾. Briefly, passage-2 human nasal epithelial cells were seeded onto a Costar Transwell (24.5 mm, 0.45-mm pore) clear culture insert (Corning Inc, Corning, NY, USA). The culture medium was changed on Day 1 and every other day until all cells were submerged. The apical medium was then removed, creating an air-liquid interface (ALI). Thereafter, the medium was changed daily, and experiments were performed using 14-day ALI grown human nasal epithelial cells. These cells served as primary HNE cells in our experiments. As needed, they were incubated in a hypoxic incubator (1% oxygen, 12 h) ⁽⁴⁾.

Concentration of cell culture supernatant

Cells (5×10^5) were seeded into 6-well plates, collecting and concentrating (Centrifugal Filter Unit; Millipore [Merck], Billerica, MA, USA) apical culture supernatants.

Western blot assay

Protein samples (30 μ g) were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Primary antibodies and horseradish peroxidase (HRP)-labeled goat anti-rabbit or goat anti-mouse immunoglobulin (Ig) secondary antibodies (Jackson Laboratory, Sacramento, CA, USA) were applied in sequence, using ECL reagent (Amersham) to develop. Relative band intensities were measured via Image J freeware (imagej.net; National Institutes of Health [NIH], Bethesda, MD, USA).

Wound-healing assay

HNE cells were grown on 12-well culture plates, using a sterile pipette tip for circular denudation (in vitro) of well centers ⁽¹¹⁾. Free cells were removed by PBS washing, and baseline photos of wound margins were obtained. The same fields were again photographed 24 hours later. Cell morphology was imaged by microscope-mounted camera for digital processing (Photoshop; Adobe Systems Inc, San Jose, CA, USA).

TEER

Transepithelial electrical resistance (TEER) was measured (Epithelial Volt-Ohm Meter [EVOM]; World Precision Instruments, Sarasota, FL, USA) as previously detailed ⁽¹³⁾. HNE cells were incubated under hypoxic conditions, immediately placing ENDOHM-12 electrodes into both apical and basal culture media. All readings represented the differences between detected apical/basal media values, expressed as standard units of ohms (Ω)/cm².

Cell viability quantification

The viability of HNE cells was based on quantifiable lactate dehydrogenase (LDH) release (CytoTox-ONE Homogeneous Membrane Integrity Assay; Promega, Madison, WI, USA), as in cited in our previous protocol ⁽⁴⁾. HNE cells were cultured (48 h) in

the presence of HMGB1 protein, and basal culture medium (50 μ L) was transferred to a separate assay plate, applying CytoTox-ONE reagent (50 μ L). A stop solution containing 10% SDS was added 10 min later, and fluorescence was measured (excitation wavelength, 560 nm; emission wavelength, 590 nm).

Immunofluorescence

Primary HNE cells were fixed (20 min, RT) in 4% paraformaldehyde, and 0.2% Triton X-100 was applied (10 min) for permeabilization. The samples were blocked (1 h, RT) in 1% bovine serum albumin (BSA)-PBS and then incubated overnight (4°C) with primary antibody in 1% BSA-PBS. Alexa Fluor 488- or 594-conjugated secondary antibody was added (1 h, RT) in sequence. Antifade mounting medium (Vectashield; Vector Laboratories Inc, Burlingame, CA, USA) was applied to complete slide preparation, using a confocal microscope (FV1000; Olympus, Tokyo, Japan) for fluorescence image capture.

Mouse experiments

Wild-type BALB/c mice (6 weeks old) were purchased (Koatech Co Ltd, Gyeonggi-do, Korea). Four animals were treated with HMGB1 (30 μ g), delivered daily (14 days) via both nostrils by 20- μ L sterile pipette (total of 420 μ g). The same volume of normal saline was administered in similar fashion to four control mice. Upon completion, the mice were sacrificed, harvesting their respiratory nasal mucosa for western blot assay and immunohistochemistry. All experiments adhered to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and were approved by the Institutional Animal Research Ethics Committee at Yonsei Medical Center.

Human samples

The institutional review board of Yonsei University College of Medicine approved this endeavor, and informed consent was granted by all participating subjects. For use in immunohistochemical and western blot analyses, we sampled the nasal polyps and middle turbinates (non-polyp nasal mucosa) of six patients with chronic rhinosinusitis (CRS) but no clinical histories of asthma, aspirin sensitivity, or cystic fibrosis. The CRS was diagnosed based on historical, endoscopic, and radiographic criteria⁽¹⁵⁾. Nasal polyps were confirmed by using nasal endoscopic findings and signs of persistent bilateral and diffuse paranasal sinus mucosal thickening in computed tomographic scans⁽¹⁶⁾. All patients had been free of steroid or antibiotics medication for at least 4 weeks before surgery. In addition, nasal lavage fluid was collected from patients with CRS in the presence (n=39) or absence (n=24) of polyps. These samples were immediately centrifuged to remove blood or mucus and stored (-70°C) until needed.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated using an established protocol⁽⁴⁾ to prepare for immunostaining (EnVision kits; Dako [Agilent], Glostrup, Denmark). After antigen retrieval and blocking, primary antibodies diluted in blocking buffer were applied for overnight incubation (4°C). Anti-rabbit Ig antibody (EnVision; Dako) was then applied and incubated (30 min). After washing in Tris-buffered saline (TBS), the slides were further incubated (20 min, RT) and ultimately colorized (10 min) using 3,3'-diaminobenzidine (DAB).

Statistics

Data were expressed as mean \pm SD. Each in vitro experiment was performed in triplicate (at minimum), analyzing outcomes via t-test or Mann-Whitney U-test. All computations relied on standard software (SPSS v20; IBM, Armonk, NY, USA), setting significance at $p < 0.05$.

Results

HMGB1 induces EMT in primary HNE cells

HMGB1 was applied to apical and basal culture media of primary HNE cells, evaluating changes in EMT markers by western blot. Expression levels of mesenchymal markers, namely vimentin and α -SMA, had increased significantly as a result (Figure 1A, B), whereas levels of epithelial markers, such as occludin, ZO-1, and e-cadherin, showed significant declines (Figure 1C, D). TEER readings in primary HNE cells were significantly lower as well (Figure 1E).

HMGB1 induces migration in primary HNE cells

As part of the EMT process, epithelial cells acquire migratory properties, and cells undergoing epithelial mesenchymal transition (EMT) are known to have a higher potential for cell motility as a consequence of cytoskeletal^(17,18). Therefore, we examined whether HMGB1 might influence HNE cellular motility. Before performing the migration and wound-healing assays, we confirmed that HMGB1 did not affect the viability of HNE cells (Supplementary Figure 2). In performing the wound scratch assay, we found that migration distance increased up to 212.5% after HMGB1 exposure, compared with controls (Figure 2A, B).

Hypoxia-induced EMT is mediated by HMGB1

It was previously reported that the development of nasal polyps is associated with hypoxic conditions⁽¹⁹⁾, and hypoxia induces EMT^(12,13,20), as well as the secretion of HMGB1 by HNE cells (Figure 3A)⁽⁴⁾. We subsequently examined the role of HMGB1 in hypoxia-induced EMT. Primary HNE cells were incubated under hypoxic conditions (1% oxygen, 12 h) with/without pre-treatment with anti-HMGB1 antibody. Western blotting revealed that levels of the epithelial marker ZO-1 declined and the mesenchymal marker vimentin increased as a result. However, anti-HMGB1

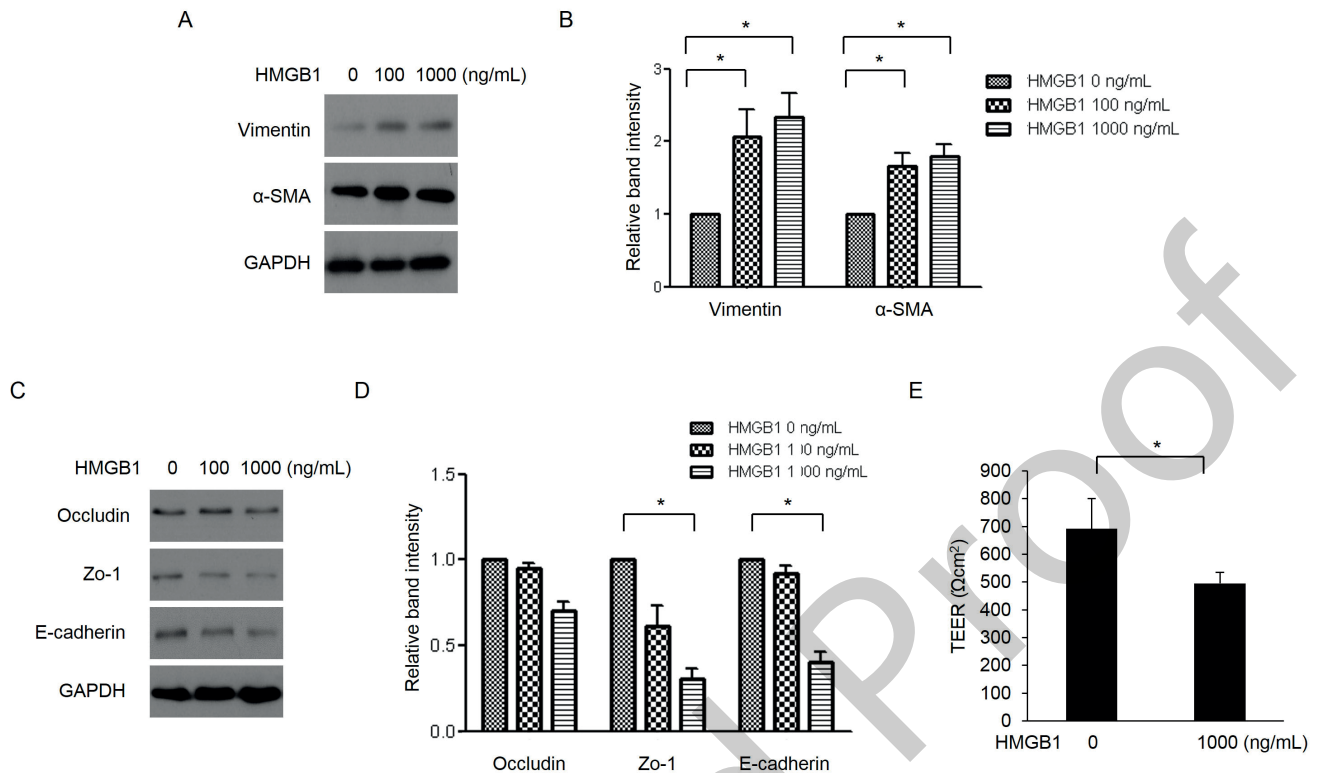


Figure 1. HMGB1 induces EMT in HNE cells. (A) Primary HNE cells were incubated (24 h) with HMGB1 yield extracts for immunoblotting (antibodies as indicated). GAPDH, loading control, SMA, smooth muscle actin; (B) Relative band intensities of vimentin and α-SMA were calculated and compared (n=3); (C) Primary HNE cells were incubated for 24 hours with HMGB1 yield extracts for immunoblotting (antibodies as indicated). GAPDH, loading control; (D) Relative band intensities of occludin, ZO-1, and E-cadherin were calculated and compared (n=3). (E) Primary HNE cells were incubated with HMGB1 for 24 hours, and TEER was measured immediately. (n=3). TEER, trans-epithelial electrical resistance.

antibody pretreatment lessened the hypoxic effects (Figure 3B, C). TEER was also diminished by hypoxia, but anti-HMGB1 antibody pretreatment similarly mitigated hypoxia-related TEER decline in HNE cells (Figure 3D). We then performed immunofluorescence studies in further support of our hypothesis, staining ZO-1 at the periphery of HNE cells. The typically even distribution of ZO-1 was disrupted and diminished by hypoxia, but anti-HMGB1 antibody pretreatment of the culture medium reduced the disruption and decreased expression of ZO-1 (Figure 4A, C). Although vimentin staining was barely visible in control HNE cells, its expression was heightened in the cytoplasm of HNE cells incubated under hypoxic conditions. Again, anti-HMGB1 antibody pretreatment dampened the augmented expression of vimentin (Figure 4B, D).

HMGB1 induces EMT through RAGE signaling

Previous studies have shown that TGF-β is a critical mediator of EMT in airway epithelial cells^(21,22). To determine if HMGB1-induced EMT is mediated via TGF-β signaling, TGF-β levels in apical and basal culture media of primary HNE cells were assayed (ELISA) with/without HMGB1 treatment. Consequently, the concentration of TGF-β was detected lower than assay range (31.2-2000 pg/mL), and we could not find significant

difference with/without HMGB1 treatment (Supplementary Figure 3A). To then ascertain if HMGB1-induced EMT is receptor dependent, we tested several cell-surface receptors, specifically the receptor for advanced glycation end products (RAGE) and Toll-like receptors (TLR2 and TLR4). Western blotting revealed an upregulation of RAGE expression 9 and 24 hours after HMGB1 treatment (1000 ng/mL), whereas expression levels of TLR2 and TLR4 proteins were unchanged (Supplementary Figure 3B, C). In addition, we incubated HNE cells with and without anti-RAGE antibody 30 min before HMGB1 treatment. The latter itself did not influence EMT marker expression; but decreased expression of ZO-1 and increased expression of vimentin due to HMGB1 were reduced by anti-RAGE antibody exposure (Figure 5A, B). In evaluating TEER, the expected HMGB1-induced decline in TEER of primary HNE cells was significantly reversed by anti-RAGE antibody pretreatment (Figure 5C). Immunofluorescence studies also revealed that anti-RAGE antibody pretreatment significantly mollifies HMGB1-related ZO-1 decline (Figure 6A) and vimentin upsurge (Figure 6B) in primary HNE cells (Figure 6C, D).

Intranasal HMGB1 induces EMT in upper respiratory epithelium of mice

We instilled HMGB1 into the nasal cavities of mice for 14 days

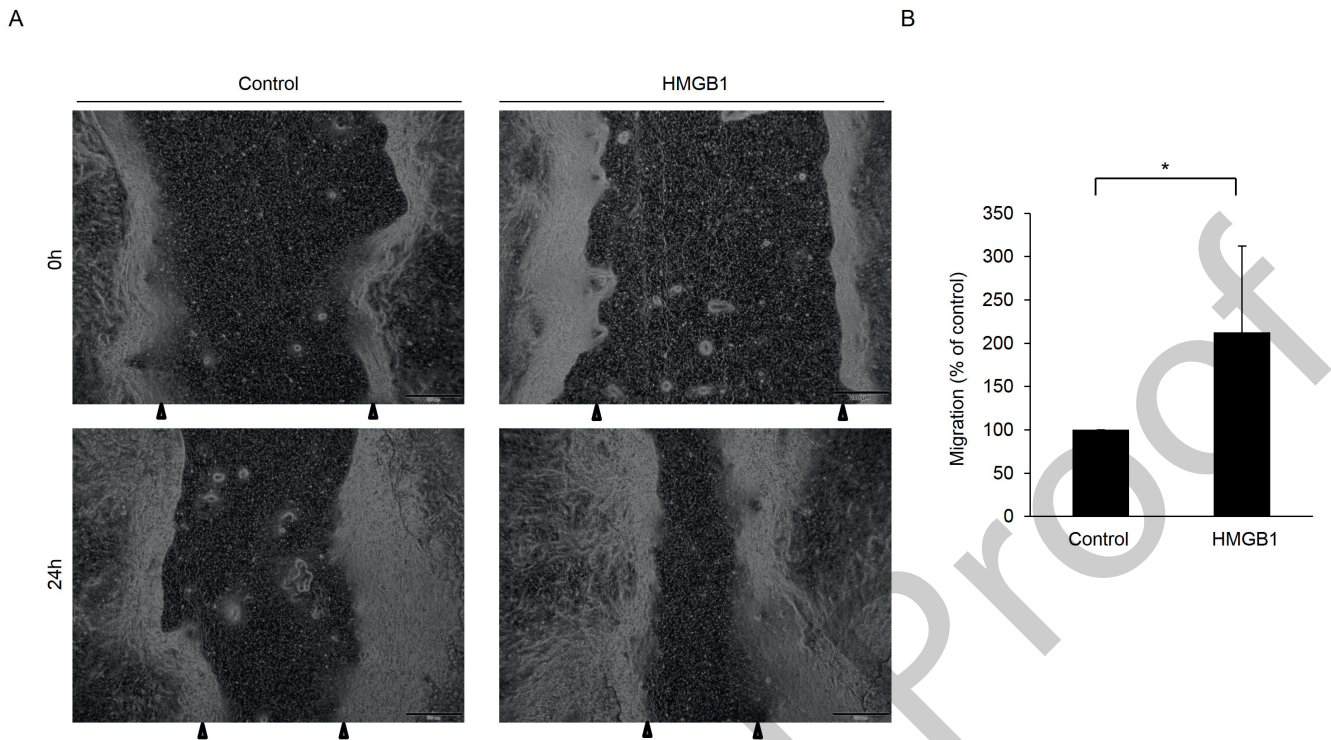


Figure 2. HMGB1 induces the migration of HNE cells: (A) Wound scratch made on Transwell culture insert of primary HNE cells, followed by incubation for 24 hours with HMGB1 (1000 ng/mL). Representative images are shown; (B) Wound closure by cells crossing into the scratch (HMGB1 treatment), compared with control (no HMGB1) was calculated using ImageJ software (n=3). Arrow indicates wound scratch.

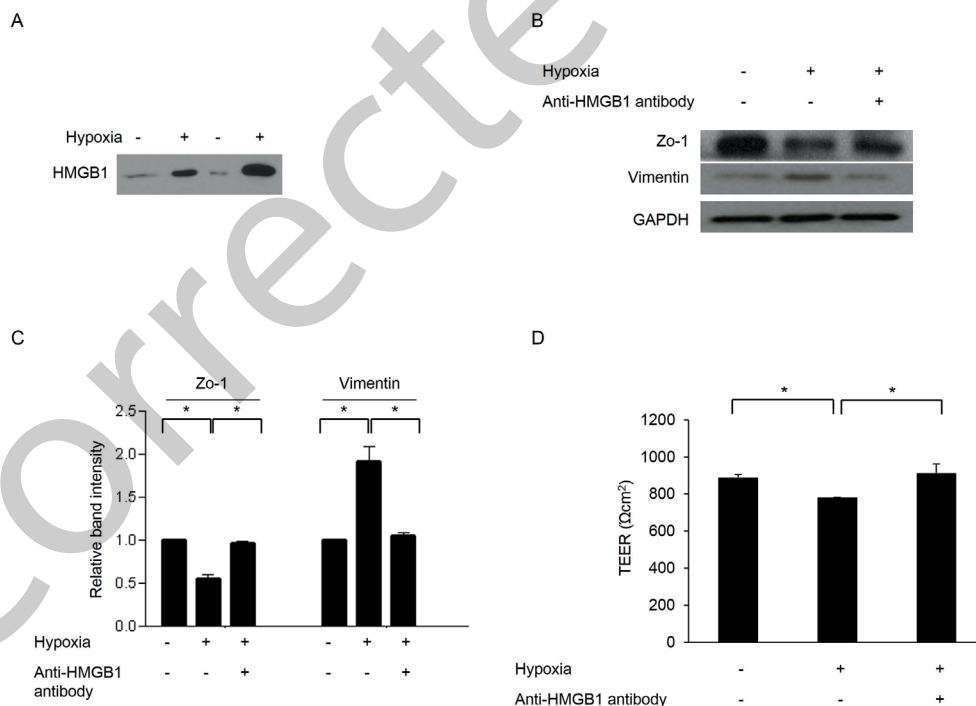


Figure 3. Hypoxia-induced EMT in primary HNE cells is dependent on HMGB1. HNE cells were incubated under hypoxic conditions with/without anti-HMGB1 antibody (1 $\mu\text{g/mL}$) pretreatment (12 h). (A) Apical supernatants of cultured HNE cells were harvested/concentrated to quantify hypoxia-induced extracellular HMGB1 protein secretion (western blot); (B) Anti-HMGB1 antibody was applied to apical and basal culture media 30 min before hypoxic incubation. Extracts were used for immunoblotting (antibodies as indicated). GAPDH, loading control; (C) Relative band intensities of ZO-1 and vimentin were calculated and compared (n=3); (D) TEER values were measured immediately and compared (n=3). TEER, trans-epithelial electrical resistance.

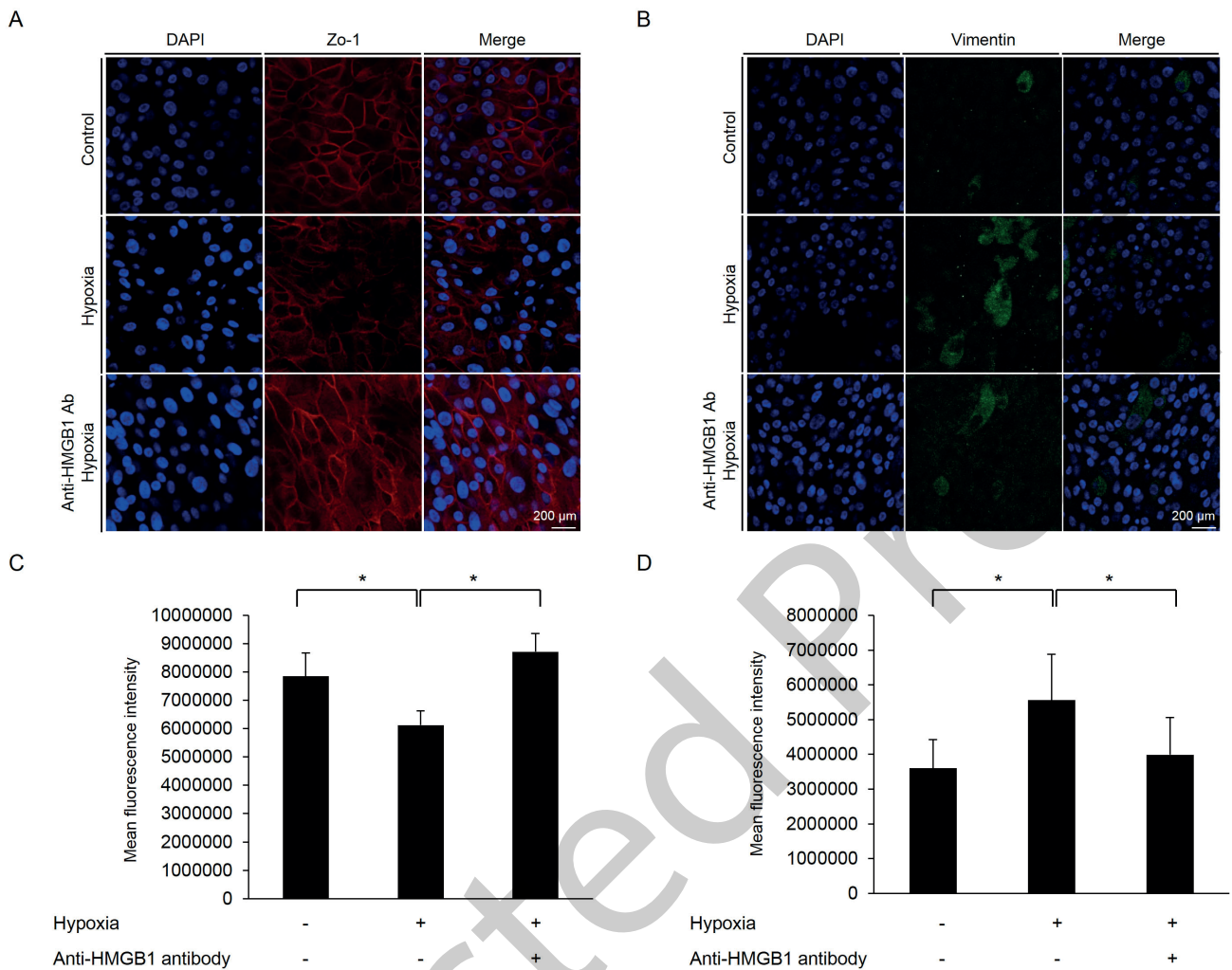


Figure 4. Hypoxia-induced EMT in primary HNE cells, dependent on HMGB1: Primary HNE cells incubated under hypoxic conditions with/without anti-HMGB1 antibody (1 μ g/mL) pretreatment. Anti-HMGB1 antibody applied to apical and basal culture media 30 min before hypoxic incubation. (A) Immunofluorescent staining with anti-ZO-1 antibody (red) and DAPI (blue) to stain DNA. Representative images shown; (B) Immunofluorescent staining of anti-vimentin antibody (green) and DAPI (blue)-stained DNA. Representative images shown; (C) Mean fluorescence intensity of ZO-1 calculated and compared via Image J software, expressed as mean \pm SD (n=3); (D) Mean fluorescence intensity of vimentin calculated and compared via Image J software, expressed as mean \pm SD (n=3).

to evaluate its effects on EMT *in vivo*. As shown by western blot, expression levels of ZO-1 in harvested nasal respiratory mucosa were subsequently lowered, whereas those of vimentin increased (Figure 7A, B, C, D); the immunohistochemical findings were similar (Figure 7E).

Cytoplasmic and extracellular levels of HMGB1 increase in nasal polyps, as do mesenchymal markers, and epithelial markers decline (relative to controls)

In our final experiment, we collected human nasal polyps, which are inherently linked to EMT⁽¹²⁾, and middle turbinate mucosa to be used as tissue controls. Relative to controls, the nasal polyps showed diminished ZO-1 and heightened vimentin expression levels; and HMGB1 level was increased by comparison (Figure 8A, B). These findings were then corroborated by immunohis-

tochemistry. Cytoplasmic expression of HMGB1 in human nasal polyps (versus controls) was much more intense, (Figure 8C). In fluids retained from human nasal polyp lavage, extracellular levels of HMGB1 exceeded those of control samples, suggesting that that HMGB1 may play a role in EMT-induced polyp formation (Figure 8D).

Discussion

The pathogenesis of nasal polyps in the setting of CRS remains unclear at present, but tissue remodeling in these lesions is believed to result from EMT and EMT-associated events (10). Here, we have found that HMGB1 induces EMT in HNE cells, propagated by an HMGB1-RAGE axis. *In vitro* studies have already shown that HMGB1-induced EMT, especially hypoxia-induced EMT, is mediated through HMGB1 signals. According to our findings,

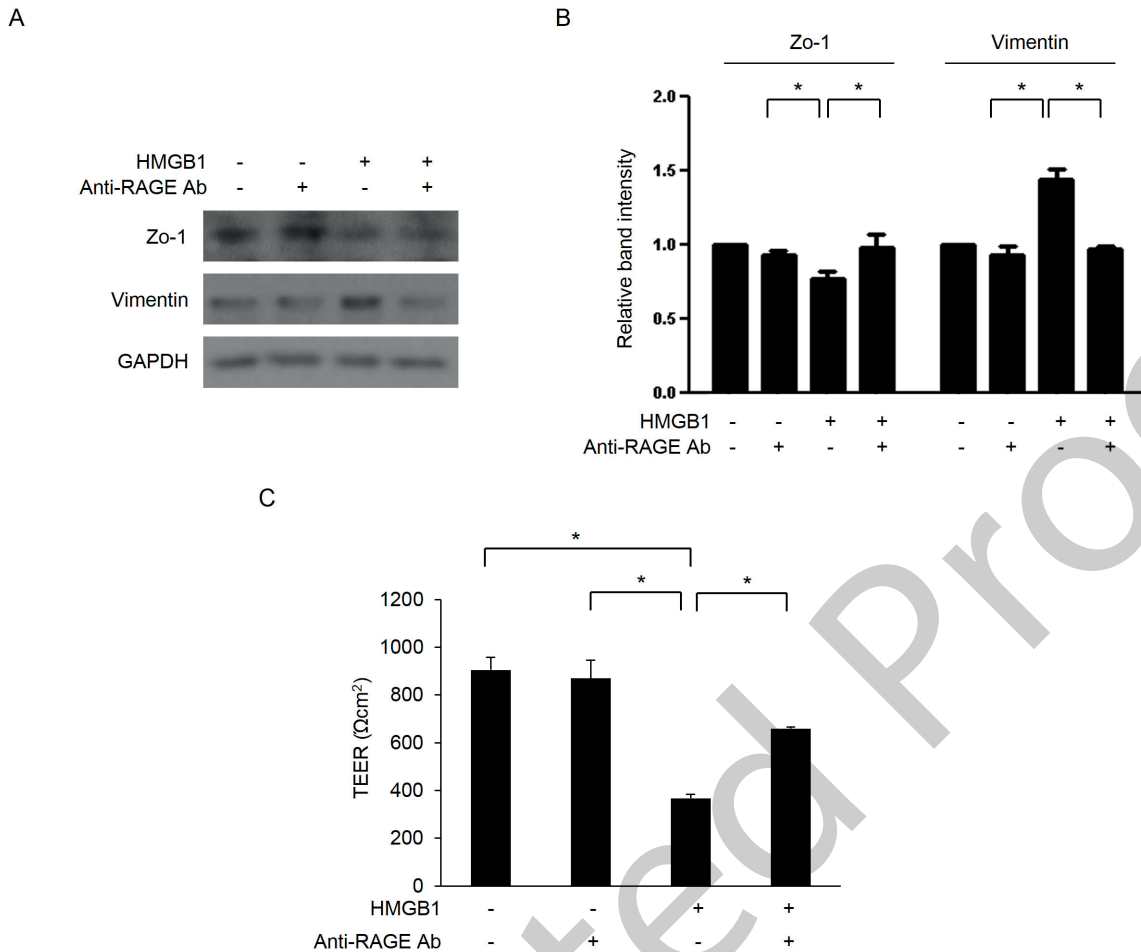


Figure 5. Western blotting to determine whether HMGB1 mediates EMT signals through RAGE. (A) Primary HNE cells were incubated for 24 hours with HMGB1 (1000 ng/mL) with/without anti-RAGE antibody (1 µg/mL) pretreatment. Anti-RAGE antibody was applied to apical and basal culture media 30 min before HMGB1 treatment. Extracts were used for immunoblotting (antibodies as indicated). GAPDH, loading control; (B) Relative band intensities of ZO-1 and vimentin were calculated and compared (n=3); (C) Primary HNE cells were incubated for 24 hours with HMGB1 (1000 ng/mL) with/without anti-RAGE antibody (1 µg/mL) pretreatment, and TEER was measured immediately. Anti-RAGE antibody was applied to apical and basal culture media 30 min before HMGB1 treatment (n=3). TEER, trans-epithelial electrical resistance.

such signaling is RAGE dependent, bearing no relation TGF-β. HMGB1 also induces an EMT-like process in mice, increasing the expression of mesenchymal markers and reducing epithelial marker expression. Our experiments using human nasal tissues and lavage fluids further indicate that relative to control mucosa, both cytoplasmic and extracellular levels of HMGB1 are increased in patients with CRS and nasal polyps. We have previously reported that hypoxia induces the secretion of extracellular HMGB1 via reactive oxygen species (ROS)-dependent mechanisms⁽⁴⁾. Another study has also confirmed that hypoxia induces EMT in HNE cells, suggesting that HIF-1α promotes nasal polyp formation via EMT⁽¹²⁾. However, HIF1-α induction of EMT in HNE cells seems to fall short when attempted, so other factors may be needed for hypoxic EMT induction in upper airways⁽¹⁰⁾. Given our past and present findings, we believe that extracellular HMGB1 secreted under hypoxic conditions may lead to EMT in HNE cells.

Extracellular HMGB1 is also known to induce lower-airway EMT. HMGB1 induction of EMT, marked by downregulation of epithelial markers and upregulation of mesenchymal markers, has been documented in A549 and RLE-6TN cells⁽⁷⁾. Although the TGF-β/p-Smad2/3 signaling pathway appears critical for HMGB1-mediated EMT in lower airways, TGF-β blockade reportedly does not confer complete inhibition⁽⁷⁾. Thus, TGF-β signaling may be not the sole pathway invested in EMT⁽⁷⁾. In the present study, HMGB1 treatment did not induce TGF-β secretion in apical and basal culture media of HNE cells (ELISA, Supplementary Figure 3A). Identical results were obtained by quantitative RNA analysis of HNE cells exposed to 1000 ng/mL of HMGB1 for 24 hours (data not shown). These findings suggest a pathway for EMT independent of TGF-β where HMGB1 may come into play. Knowledge of normal physiologic HMGB1 levels is essential to properly define the role of EMT in human nasal mucosa. At least one report has maintained that 48-hour exposure to HMGB1 (10

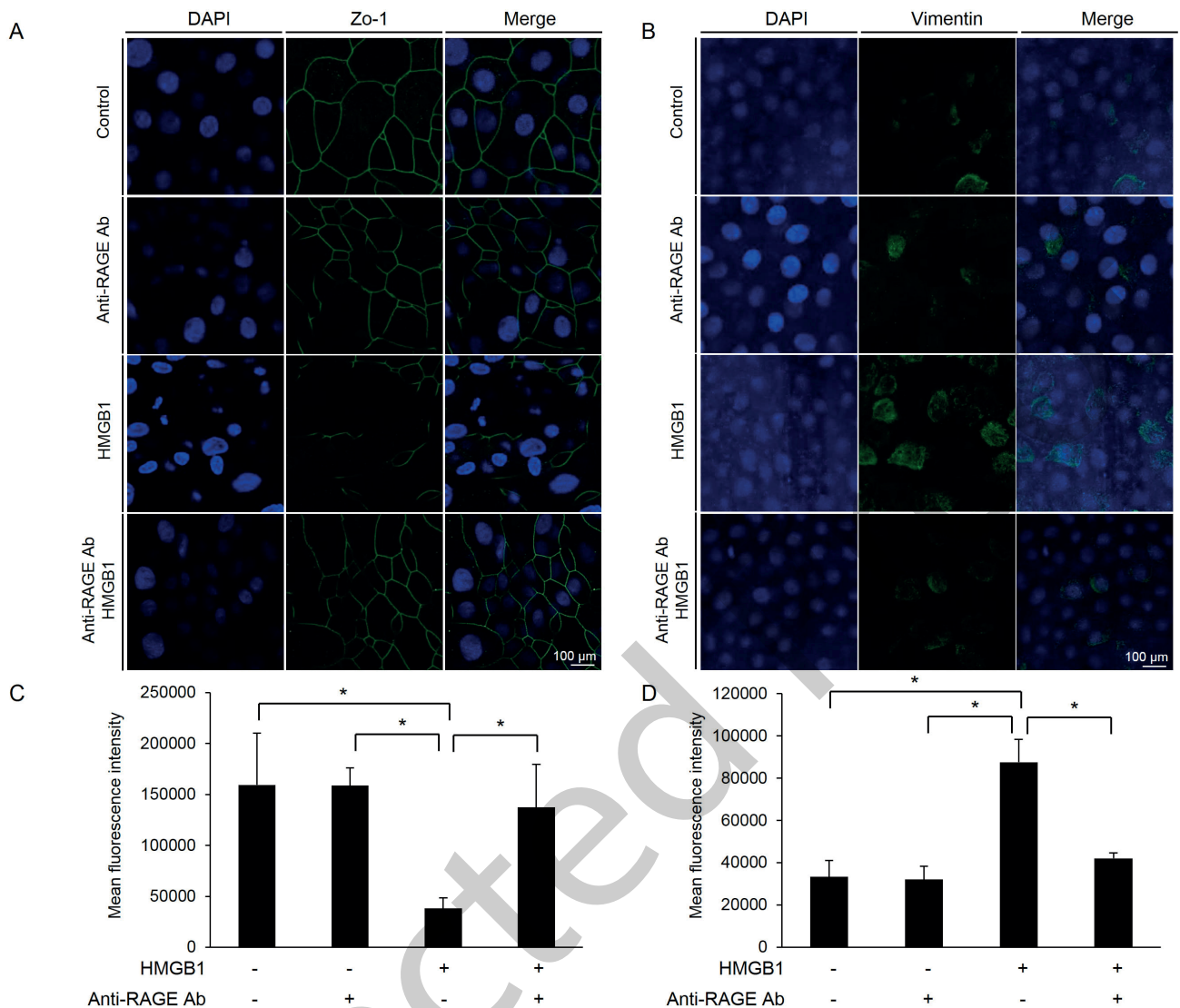


Figure 6. Immunofluorescence analysis to determine whether HMGB1 mediates EMT signals through RAGE. Primary HNE cells were incubated for 24 hours with HMGB1 (1000 ng/mL) with/without anti-RAGE antibody (1 μ g/mL) pretreatment. Anti-RAGE antibody was applied to apical and basal culture media 30 min before HMGB1 treatment. (A) Immunofluorescent staining with anti-ZO-1 antibody (green) and DAPI (blue). Representative images are shown; (B) Immunofluorescent staining with anti-vimentin antibody (green) and DAPI (blue). Representative images are shown; (C) Mean fluorescence intensity of ZO-1 was calculated and compared via Image J software, and expressed as mean \pm SD (n=3); (D) Mean fluorescence intensity of vimentin was calculated and compared via Image J software, and expressed as mean \pm SD (n=3).

μ g/mL) seems sufficient to induce morphologic and biochemical features of EMT in lower airway epithelial cells⁽⁸⁾. As for the present investigation, we added 1 μ g/mL of HMGB1 to both apical and basal culture media. In the absence of established relevant norms for upper airways, a mean HMGB1 concentration of 160 ng/mL was recorded through human nasal lavage fluids analysis⁽²³⁾. Because the effects of HMGB1 may differ as concentrations vary, it is feasible that other pathways are activated accordingly, producing a host of manifestations^(24,25). Furthermore, the level of HMGB1 in healthy controls was higher enough to induce the effects of HMGB1 observed in in vitro experiments. As redox status of HMGB1 is important in determining the function of

HMGB1⁽²⁶⁾, and nasal obstruction could change the oxidative stress in nasal cavity⁽²⁷⁾, we hypothesized that differential environmental condition such as redox status also might affect the function of HMGB1. Precise measures of extracellular HMGB1 protein in large-scale studies of human upper airways are then mandatory to clarify the role of HMGB1 in EMT or in nasal polyp formation.

Despite the often banal perceptions of nasal polyps, a greater burden of illness is exacted by their clinical refractoriness^(12,28). Previous evidence has shown that EMT is crucial in the development of human nasal polyps⁽¹²⁾, prompting our patient-based examinations. We found that HMGB1 levels were higher in ex-

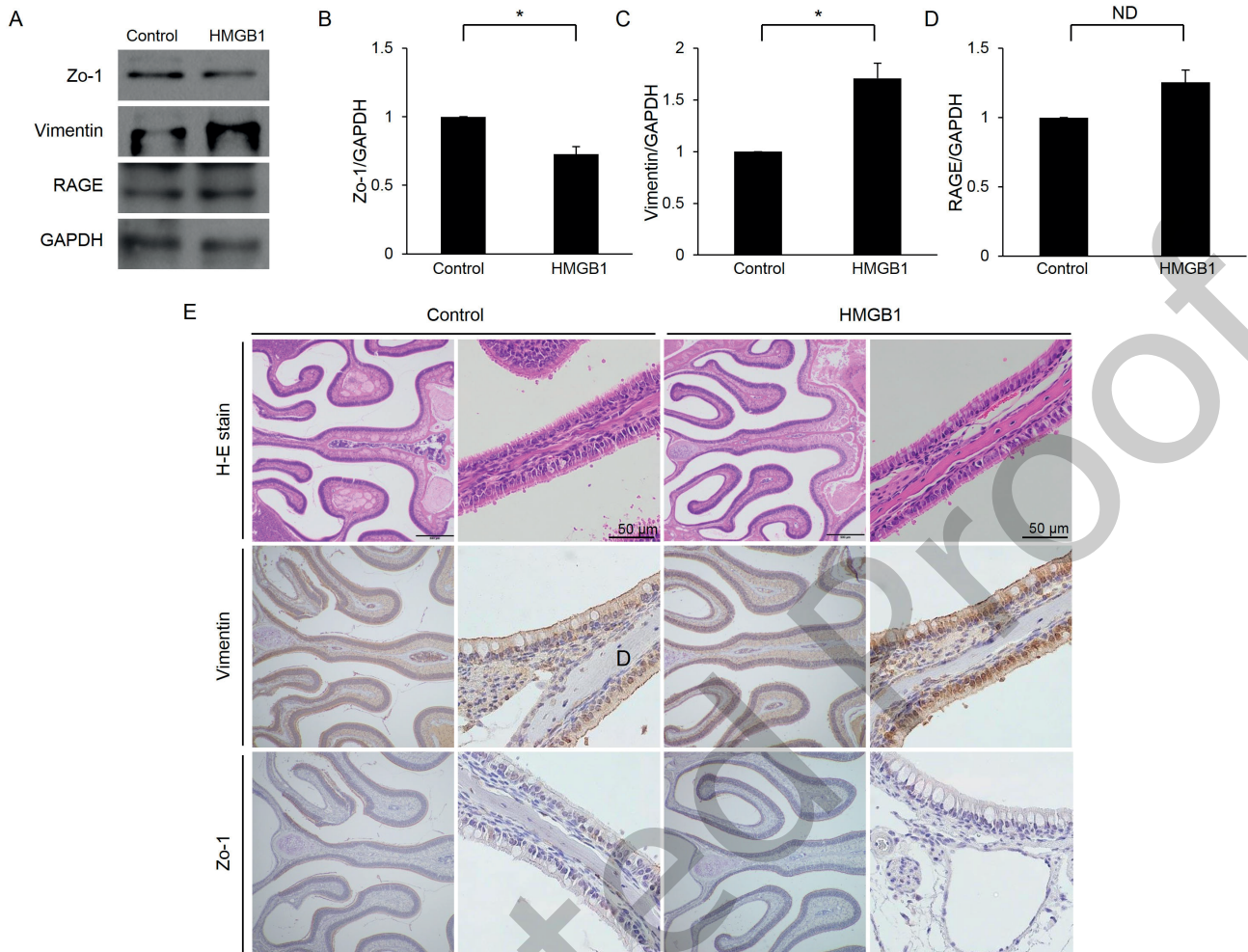


Figure 7. Intranasal HMGB1 decreases ZO-1 level and increases vimentin level in BALB/c mice. HMGB1 (30 μ g) was instilled daily (14 days) in both nostrils of four wild-type BALB/c mice, and PBS alone was instilled in four wild-type BALB/c control mice. Nasal respiratory mucosa were harvested thereafter. (A) Western blot of mouse mucosal lysates (antibodies as indicated). GAPDH, loading control; (B-D) Relative band intensities of ZO-1, vimentin, and RAGE were calculated and compared (n=4); (E) Immunohistochemical stain of anti-vimentin and anti-ZO-1 antibodies. Routine H & E staining is also shown.

tranuclear compartments (especially cellular cytoplasm) and in lavage fluid from nasal polyps, compared with control mucosa. Also, our western blot and immunohistochemical results mirrored findings of prior studies, showing diminished epithelial and heightened mesenchymal marker expression in the presence of human nasal polyps.

Although an association between HMGB1 and nasal polyps has been previously reported, no publication to date has addressed HMGB1-related mechanisms within upper respiratory epithelium^(29,30). From our perspective, this is the first study aimed at the role of HMGB1 in EMT and its ramifications for nasal polyp formation.

Our study has several acknowledged limitations. First, we did not check for allergic rhinitis in our human participants. Although control subjects and sufferers of allergic rhinitis have displayed no significant differences in epithelial and mesenchymal markers⁽⁹⁾, eosinophils do contribute to the pathogenesis of

nasal polyps in CRS⁽³¹⁾, so the potential effects of allergic rhinitis should be researched. Furthermore, we did not observe nasal polyp formation after 2 weeks of HMGB1 treatment in mice, so we have no direct evidence that HMGB1 induces nasal polyps. Third, we cultured primary nasal epithelial cells obtained from inferior turbinate mucosa, and performed *in vitro* experiments. In our previous studies, we proved that our protocol using ALI system with retinoic acid induces secretory and mucociliary re-differentiation similar to normal human nasal epithelial cells^(14,32). Therefore, our *in vitro* experiments are suggestive the effect of HMGB1 on normal nasal epithelial cells, and do not suggest the effect of HMGB1 on nasal epithelial cells of polyps. In a recent study, the epithelium from nasal polyps and middle turbinates showed different genetic profiles⁽³³⁾. Therefore, our *in vitro* findings could be different in epithelium originated from nasal polyps. Finally, we did not perform *in vivo* inhibition to block HMGB1 functions. Despite current data suggesting that

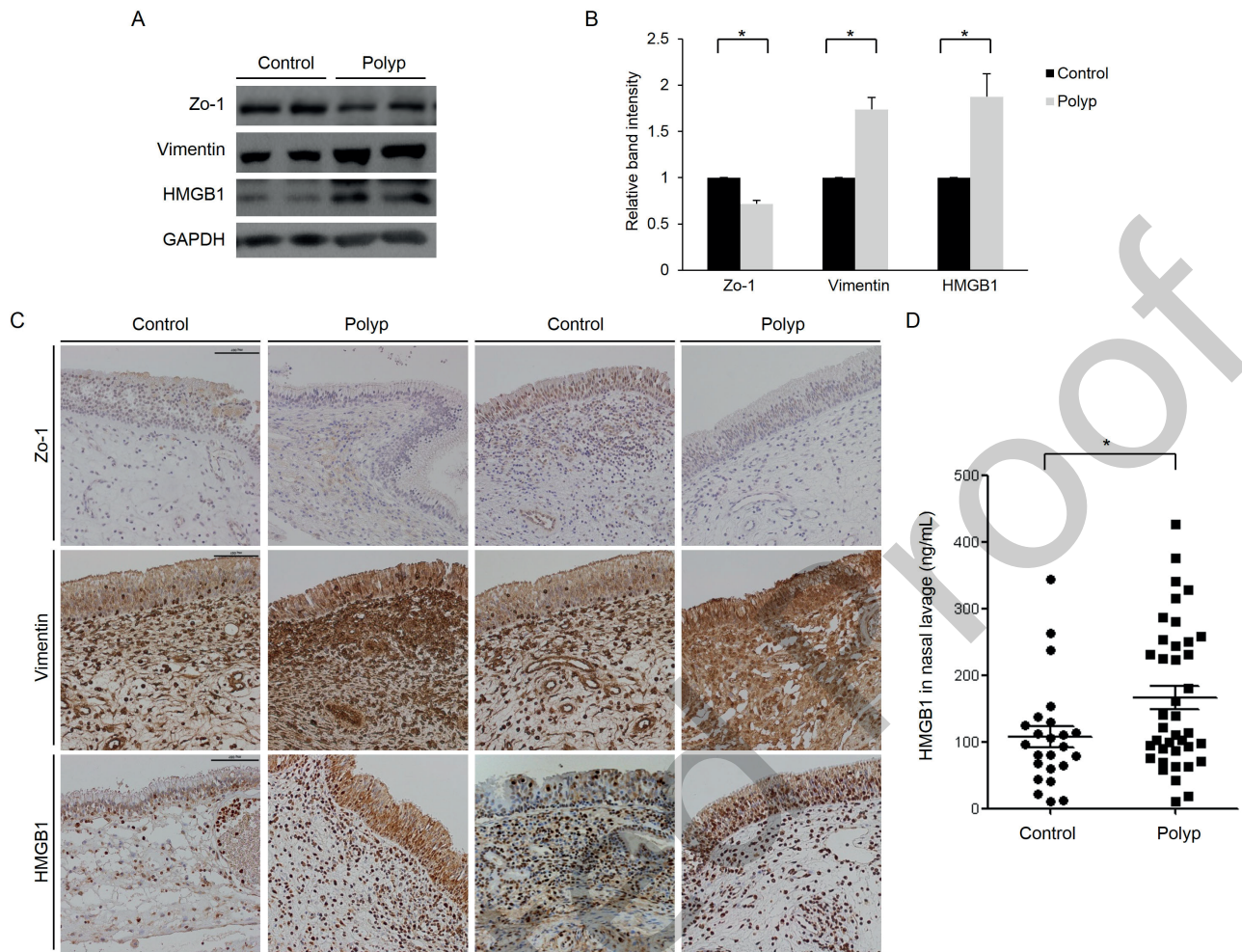


Figure 8. HMGB1 is increased in nasal polyps, relative to control. (A) Nasal polyps (n=6) and control tissues (n=6) were collected from patients for western blot of ZO-1, vimentin, and HMGB1 expression levels. GAPDH, loading control; (B) Relative band intensities of ZO-1, vimentin, and HMGB1 were calculated; (C) Immunohistochemical staining of ZO-1, vimentin, and HMGB1 expressed in human nasal polyps, compared with controls (n=3); (D) HMGB1 was assayed using ELISA in human nasal lavage fluids, to compare HMGB1 expression in human nasal polyps (n=24) and controls (n=39).

HMGB1 induces EMT in HNE cells, the protective role of HMGB1 inhibition in EMT and nasal polyp formation awaits further characterization, upon which therapeutic potential may ultimately hinge.

Conclusion

In conclusion, we provide the first evidence of HMGB1/RAGE-mediated EMT in HNE cells, independent of TGF- β , confirming the role of HMGB1 in hypoxia-induced EMT. The heightened expression and extracellular release of HMGB1 inherent in human nasal polyps were also demonstrated. These findings suggest that HMGB1 may hold therapeutic potential in treating patients with CRS and nasal polyps.

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Authorship contribution

Performed experiments: H.J.Min, J.W.Cho; Wrote the manuscript: H.J.Min, K.S.Kim; Supervised the study: J.H.Yoon, C.H.Kim; All authors participated in data interpretation.

Conflict of interest

None.

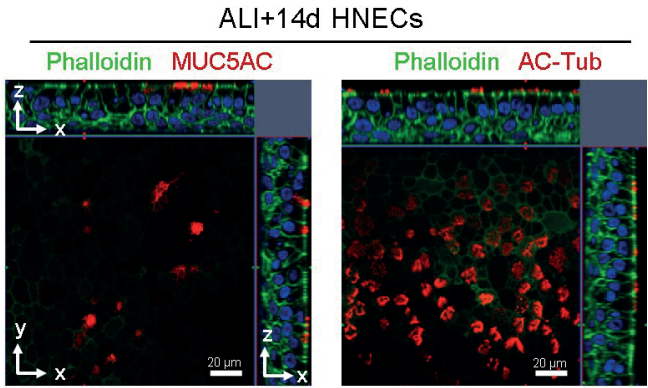
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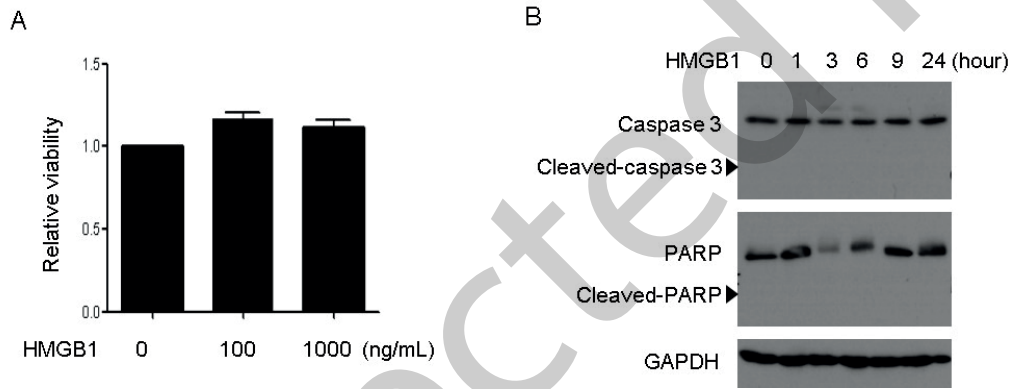
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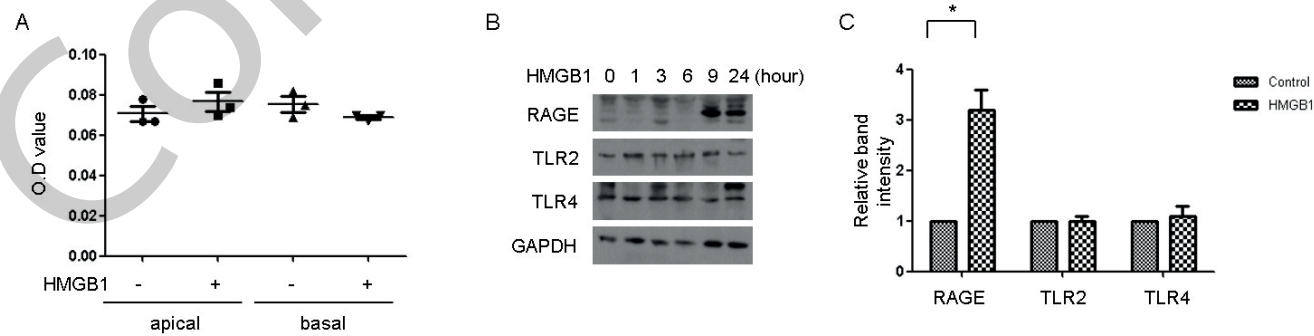
SUPPLEMENTARY INFORMATION



Supplementary figure 1. Representative confocal microscope images of HNECs differentiated at the ALI culture for 2 weeks. Cells were stained for the secretory cell marker MUC5AC (red) and the ciliated cell marker acetylated α -tubulin (Ac-Tub; red), and counterstained with DAPI (blue) and FITC-phalloidin for F-actin (green). Immunofluorescence images show xy and xz sections. Scale bar, 20 μ m.



Supplementary figure 2. Viability of HNE cells are not influenced by HMGB1. (A) Primary HNE cells were incubated with HMGB1 (concentrations as indicated), and LDH release assay was performed to determine cell viability; (B) Primary HNE cells were incubated with HMGB1 (1000 ng/mL) for durations indicated, and anti-caspase3 and PARP were assayed using western blot to evaluate the effect of HMGB1 on cell apoptosis.



Supplementary figure 3. HMGB1 upregulates RAGE expression but not that of TGF- β in primary HNE cells. Primary HNE cells incubated for 24 hours with HMGB1 (1000 ng/mL). (A) Apical and basal culture media harvested for TGF- β assay (ELISA); (B) Extracts used for immunoblotting, determining expression levels of RAGE, TLR2, and TLR4. (C) Relative band intensity at 24 hours after treatment was calculated and compared. GAPDH, loading control, RAGE, receptor for glycation end product, TLR, Toll-like receptor.