



Phosphorylation of low density lipoprotein receptor-related protein 6 is involved in receptor for advanced glycation end product-mediated β -catenin stabilization in a toluene diisocyanate-induced asthma model

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ABSTRACT

Background: We have previously demonstrated that the receptor for advanced glycation end products (RAGE)/ β -catenin axis plays a vital role in regulating airway inflammation and airway remodeling in a toluene diisocyanate (TDI)-induced murine asthma model. However, the exact mechanism of β -catenin activation remains unclear. Given that phosphorylation of the low-density lipoprotein receptor-related protein 6 (Lrp6) is a key step in mediating β -catenin stabilization in canonical wnt/ β -catenin signaling, we explored the possible relationship between RAGE and Lrp6 in regulating β -catenin stabilization in TDI-induced asthma.

Methods: In this study, a TDI-induced murine asthma model was generated, and mice were treated with a specific inhibitor of RAGE. In vitro, the human bronchial epithelial cell line 16HBE was treated with TDI-human serum albumin (TDI-HSA). RAGE overexpression or knockdown cells were also constructed and assessed.

Results: The results showed that RAGE inhibition or RAGE knockdown decreased β -catenin nuclear accumulation and the expression of relevant β -catenin targeted genes (VEGF, MMP9, TGF- β 1) in the TDI-induced murine asthma model and TDI-HSA-treated 16HBE cells, respectively. Silencing of RAGE reversed the TDI-induced increase in phospho-ERK1/2 (p-ERK) and phospho-Lrp6 (p-Lrp6) in 16HBE cells. Pretreatment with the extracellular signal-regulated kinase (ERK)1/2 inhibitor U0126 suppressed TDI-induced Lrp6 phosphorylation. Furthermore, knockdown of Lrp6 in 16HBE cells decreased β -catenin nuclear translocation and the expression of VEGF, MMP9, and TGF- β 1.

Conclusion: These data suggested that the RAGE/ERK axis modulates Lrp6 phosphorylation, contributing to β -catenin stabilization in a TDI-induced murine model.

1. Introduction

Toluene diisocyanate, a widely used chemical in many industrialized areas, is the most commonly reported cause in occupational asthma (OA), which has been reported in large epidemiologic studies to account for up to 10% to 25% of new-onset adult asthma [1]. In recent decades, relatively few studies have been undertaken to determine the mechanisms of toluene diisocyanate (TDI)-mediated airway inflammation. Previously, we demonstrated aberrant expression of β -catenin in a TDI-induced asthma model, and found that selective blockade of β -catenin-mediated transcription via ICG-001 and XAV-939 significantly relieved TDI-induced airway remodeling and inflammation [2]. However, the upstream mechanism regarding activation of β -catenin signaling in this TDI-induced asthma model has not been fully elucidated.

In mature epithelial cells, β -catenin is an evolutionary conserved

molecule that exerts dual roles in cellular signaling. It is an integral structural component of cadherin-based adherens junctions and the key nuclear effector of canonical wnt signaling in the nucleus [3]. In the absence of wnt signals, cytosolic β -catenin binds to a multiprotein destruction complex including axin, glycogen synthase kinase 3 β (GSK3 β), and adenomatous polyposis coli (APC), which marks β -catenin for ubiquitin-mediated degradation by the 26S proteasome [4]. Upon wnt stimulation, wnt ligands bind to their membrane co-receptors, Frizzled and the low-density lipoprotein receptor-related protein 6 (Lrp6). This leads to β -catenin stabilization and translocation to the nucleus, which stimulates the transcription of target genes in cooperation with T-cell factor/lymphoid enhancer-binding factor [3], regulating various biologic processes, such as organ development, tissue homeostasis, and pathogenesis of human diseases.

Phosphorylation of the Lrp6 intracellular domain is a key step in the

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transmembrane transduction of wnt signals, which initiate the downstream cascade that leads to β -catenin stabilization [5]. Lrp6 is a highly modular, single-pass transmembrane protein, and its extracellular domain mediates the interaction between Lrp6 and extracellular ligands, such as wnts and dickkopf (Dkk1) [6,7]. The intracellular domain of Lrp6 is comprised of five P-P-[S/T]-P(PPSP) repeats and juxtaposed casein kinase 1 (CK1) sites, which are referred to as PPSPXS motifs. Efficient phosphorylation of intracellular site is required for signalosome formation, Axin recruitment, and direct inhibition of GSK3, which are essential for subsequent β -catenin stabilization and target gene transcription [8–12]. Lrp6 phosphorylation can be mediated by multiple kinases, depending on the stimuli and cellular context. Zeng et al. identified GSK3 and CK1 as dual kinases that mediate wnt-dependent Lrp6 phosphorylation [13]. Recently, mitogen-activated protein kinases (MAPKs) have also been shown to phosphorylate Lrp6 following fibroblast growth factor (Fgf) and hepatocyte growth factor (Hgf) stimulation [14,15].

The receptor for advanced glycation end products (RAGE) is a multiligand pattern recognition receptor and can be stimulated by a diverse range of pathogen- and host-derived endogenous ligands, including advanced glycation end products (AGEs), high mobility group box-1 protein (HMGB1) and others, initiating a number of physiological and pathological events characterized by inflammatory responses. It is well established that RAGE engagement can activate a complex array of signaling pathways, including MAPK pathways and GSK3 β [16]. Interestingly, we previously demonstrated that RAGE is required for β -catenin stabilization in a TDI-induced murine asthma model [17]. In vitro, it was recently reported that HMGB1 treatment induced Lrp6 phosphorylation (p-Lrp6) at Ser1490 in BEAS-2B cells [18].

In this study, we hypothesize that RAGE is involved in the phosphorylation of Lrp6 and mediates β -catenin stabilization during TDI-induced asthma. Our findings indicated that Lrp6 plays a vital role in mediating TDI-induced β -catenin stabilization in bronchial epithelial cells and that this involves RAGE/ERK axis-mediated Lrp6 phosphorylation.

2. Materials and methods

2.1. Reagents

TDI (toluene-2,4-diisocyanate, $\geq 98.0\%$) was obtained from Sigma-Aldrich (Shanghai, China). The vehicle(AOO) used to dissolve TDI consisted of a mixture of 2 volumes of acetone and 3 volumes of olive oil for sensitization, and 1 volume of acetone and 4 volumes of olive oil for the challenge. The RAGE inhibitor FPS-ZM1, ERK inhibitor U0126 was purchased from Selleck Chemicals. Antibodies used are as follows: rabbit anti- β -catenin (A11932 1:1000), anti-VEGF (A12303 1:1000), anti-MMP9 (A2095 1:1000), and anti-TGF- β 1 (A2124 1:1000) were bought from Abclonal Biotech (Beijing, China). Rabbit anti-phospho-Lrp6 (Ser1490) (#2568 1:500), anti-non-phospho(Active) β -catenin (#19807 1:1000), anti-total-ERK1/2 (#4695 1:1000), anti-phospho-ERK (p-ERK) (#4370 1:1000), anti-p38(#8690 1:1000), and anti-phospho-p38(#4511 1:1000) were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-Lrp6 (ab134146 1:500), and anti-RAGE (ab3611 1:1000) were from Abcam.

2.2. Animals and experimental protocol

Specific-pathogen-free male BALB/c mice (20–22 g) aged 6–8 weeks were purchased from Southern Medical University. The mice were housed in a specific pathogen-free facility and fed with sterile water and irradiated food ad libitum. All studies were conducted in accordance with the committee of the Southern Medical University on the use and care of animals, and the protocols were approved by the Animal Subjects Committee of Nanfang Hospital. Mice were randomized into the following four groups: (1) control group; (2) AOO (the vehicle used

to dissolve TDI) group; (3) TDI group; and (4) FPS-ZM1 group. Animal models were generated as described previously [17]. Briefly, on days 1 and 8, the mice were dermally treated with 0.3% TDI on the dorsum of both ears (20 μ L/ear). On days 15, 18, and 21 the mice were placed in a horizontal rectangle chamber and challenged for 3 h each time via the airways with 3% TDI by means of compressed air nebulization. An ultrasonic nebulizer (NE-C28; Omron, Tokyo, Japan) driven by compressed air was used to aerosolize TDI, with operating air flow rate of 3.5 L/min and mass median aerodynamic diameter (MMAD) of 3 mm. As a control, mice were sensitized and challenged by the same procedures with the same amount of AOO. FPS-ZM1 (1.5 mg/kg; i.p.) dissolved in DMSO and diluted with PBS (pH = 7.4) was given to the mice immediately after each airway challenge.

2.3. Assessment of airway hyperresponsiveness (AHR), preparing lymph node cells and analysis of bronchoalveolar lavage fluid (BALF)

Twenty-four hours after the last inhalation, mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Troy, NY) and challenged with increasing doses of methacholine (2.5, 5, 10 mg/mL) by means of ultrasonic nebulization. Measurements of lung resistance (RL) were expressed as percentage of baseline RL value for each concentration of methacholine.

After measuring airway parameters, the mice were sacrificed with pentobarbital (100 mg/kg i.p. weight). Superficial parotid lymph nodes were dissected from the mice, which were processed for each mouse separately. As previously described [19], The lymph nodes were kept on ice in RPMI-1640 medium, and cell suspensions were obtained by pressing the lymph nodes through a 100 μ m cell strainer and rinsing with 10 mL tissue culture medium (RPMI-1640). Cells were seeded into 48-well culture plates at a density of 10^6 cells/mL and incubated in complete tissue culture medium (RPMI-1640 supplemented with 10% heat-inactivated FBS) for 43 h with 5 mg/mL of concanavaline A (Sigma-Aldrich, Shanghai, China), then centrifuged and the supernatants were stored at -80°C for further measurement of IL-4 by ELISA (BoShiDe Biological Engineering Company, Wuhan, China). After superficial parotid lymph nodes were obtained, PBS was infused slowly into the lungs and withdrawn via a cannula inserted into the trachea to obtain BALF. For differential cell counts, a cytospin sample (Shandon Scientific, Runcorn, UK) was prepared and stained using hematoxylin and eosin (H&E) (Nanjing Jiancheng, China). For each sample, a total of 200 cells were counted for the numbers of macrophages, eosinophils, neutrophils and lymphocytes.

2.4. Lung tissue for western blotting, and immunohistochemistry

Mice were humanely euthanized with pentobarbital and lungs removed. The left lung was infused with 4% formaldehyde to fully inflate all lobes (judged visually), the lungs were kept in paraformaldehyde for at least 6 h, and then fixed and embedded in paraffin. Lung sections (4 μ m) were cut with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The right lung was removed and stored at -80°C . For immunohistochemistry of β -catenin, lung tissue on slides was treated with 0.3% H_2O_2 for 10 min to block endogenous peroxidase and then incubated 4°C overnight with anti- β -catenin (A11932 1:200 Abclonal) antibody. After incubation with peroxidase conjugated secondary antibody, signals were visualized with a DAB peroxidase substrate kit. For western blotting analysis, whole lung extracts mixed with $5 \times$ SDS loading buffer were separated by 10% SDS-polyacrylamide gel and transferred onto PVDF membranes (Millipore, Bedford, MA), and then probed with anti-RAGE, anti-Lrp6, anti-phospho Lrp6 (S1490), anti-MMP9, anti-VEGF and anti-TGF- β 1 antibodies with indicated dilutions. After incubation with IRDye $^{\text{®}}$ 680WC-conjugated secondary antibody, immunoreactive bands were exposed to Odyssey $^{\text{®}}$ CLx Imager (LI-COR Biosciences, Lincoln, NE) for image capture. Quantitative image analysis was performed with Image

J software.

2.5. Preparation of TDI-human serum albumin (TDI-HSA) conjugates

TDI-HSA conjugates were prepared as previously described [2].

2.6. Stable RAGE knockdown and overexpression in 16HBE cells, cell culture, transfection, and inhibitor treatments

Lentiviral systems for knocking down and overexpressing the RAGE gene were completed by ABM Biotechnology Co. (Nanjing, China). Lentiviral plasmids against RAGE proteins were constructed. These constructs and an empty vector were used to transfect 16HBE cells (Shanghai Fuxiang Biological Technology Co., ATCC, Portland, OR, USA). The transfected 16HBE cells were named the RAGE-shRNA group, RAGE-cDNA group, and Vector group, respectively. The normal 16HBE cells were designated the wild-type (WT) group or control group. Silencing and overexpression of the RAGE protein was determined via Western blot analysis. The Lrp6 siRNA sequence was sense: 5'-ACAUGUUCUGCAGUAGA-3', antisense: 5'-UCUAAACUGCA GAACAAUGU-3', and siRNA-mediated knockdown of Lrp6 in 16HBE cells was performed using Lipofectamine 2000 transfection reagent (Life Technologies, Gaithersburg, MD, USA). Lrp6 siRNA was transfected into 70% confluent 16HBE cells according to the manufacturer's protocol. Cells were cultured in DMEM (GinuoBiopharm Technology Co., Shanghai, China) with 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA) and placed in a humidified incubator at 37 °C with 5% CO₂. When reaching 90% confluence, the cells were seeded to proper culture plates, different concentrations of TDI-HSA conjugates, or recombinant human wnt3a (R&D systems) protein were added to the culture medium for indicated times. For ERK inhibition studies, 16HBE cells were pretreated with inhibitor for 2 h.

2.7. Western blot analysis and immunofluorescence

To evaluate the protein expression of p-Lrp6, β -catenin and the activation of signaling pathway in vitro. Total cell lysates were obtained according to manufacturer's instructions (KeyGen Biotech, Nanjing, China). The protein products were subjected to 10% or 12% SDS PAGE, transferred to PVDF membrane (Millipore), and probed with antibodies as follows: anti-Lrp6, anti-p-Lrp6 (S1490), anti-active- β -catenin, anti-p-ERK1/2, anti-t-ERK1/2, anti-p38, anti-p-p38 with indicated dilutions. After incubation with IRDye® 680WC-conjugated secondary antibody, the membranes were visualized by Odyssey® CLx Imager. Quantitative image analysis was performed with Image J software.

In a parallel experiment, collected cells were fixed with a 4% paraformaldehyde solution and permeabilized with 0.1% Triton X-100. Then, the cells were blocked with 5% bovine serum albumin in PBS. All samples were subsequently incubated with monoclonal anti- β -catenin antibody overnight at 4 °C with following incubation with FITC (green)-linked anti-rabbit IgG for 30 min at room temperature. Cell nuclei were labeled with DAPI (2 μ g/ml) for 20 min and then the cells were rinsed for three times. A laser-scanning confocal microscope (Olympus, Tokyo, Japan) was used to examine the distribution of β -catenin in 16HBE cells.

2.8. Nuclear and cytoplasmic protein extraction

Stable RAGE knockdown cells and empty vector cells as well as 16HBE cells which transfected with the Lrp6 siRNA or control siRNA were respectively cultured in DMEM containing 10% (v/v) FBS with or without TDI-HSA (80 μ g/ml) treatment for 12 h. Then cells were trypsinized, washed with ice-cold PBS, and centrifuged according to the manufacturer's protocol (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Thermo Scientific, Logan, UT, USA). Fresh protease and phosphatase inhibitors (KeyGen Biotech, Nanjing, China) were added to

the manufacturer's reagents. The cytoplasmic and nuclear extracts were analyzed via Western blot.

2.9. Real-time reverse transcription polymerase chain reaction (RT-PCR)

Expression of several wnts, MMP9, VEGF, and TGF- β 1 were detected via the SYBR green-based PCR technique. Total lung tissue or cellular RNA was isolated using an RNAiso Plus kit (Takara, Guangzhou, China). RNA samples were then reverse-transcribed into first-strand cDNA using the Prime-Script RT reagent kit from Takara. A model LightCycler 480 Fast Real-Time PCR System was used. Primers for amplifying wnt3a: 5'-CCCTGTTCTGGACAAAGCCAC-3' (sense), 5'-GCTGCTGCACCCACAGATAG-3' (antisense). wnt7b: 5'-ACAGGAGGG TGGGGATAGA-3' (sense), 5'-GAAACAGCCAGGAAACCGT-3' (antisense). wnt10b: 5'-TCAGTCGGGCTTAAGCAAT-3' (sense), 5'-TG GTG TGACACTCGTGAAC-3' (antisense). MMP9: 5'-CGCCTCTGGAGGTTT CAC-3' (sense), 5'-AACTCACGCGCCAGTAGAAG-3' (antisense). GAPDH: 5'-AGTGGATATTGTTGCCATCA-3' (sense), 5'-GAAGATGGTG ATGGGATTTC-3' (antisense), TGF- β 1: 5'-CGACTCGCCAGAGTGGT TAT-3' (sense), 5'-CGGTAGTGAACCCGTTGATGT-3' (antisense), VEGF: 5'-ATCTTCAAGCCATCCTGTGTGC-3' (sense), 5'-CAAGGCCACAGGGA TTTTC-3' (antisense).

2.10. Statistical methods

SPSS 20.0 software (SPSS, Chicago, IL, USA) was used for the statistical analyses. Statistical analysis was performed via one-way analysis of variance (ANOVA), and post hoc tests were analyzed by Bonferroni (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc tests for multiple comparisons. Results were presented as mean $\pm$ standard deviations (SD). $p < 0.05$ was considered to be statistically significant. At least three independent experiments were repeated.

3. Results

3.1. RAGE inhibitor decreases phosphorylation of Lrp6 and ameliorates redistribution of β -catenin and β -catenin-mediated gene expression in a TDI-induced murine asthma model

We first assessed the level of phosphorylation of Lrp6 and the expression of several wnts, as well as expression of β -catenin and relevant target genes, in the TDI-induced murine asthma model. To demonstrate that asthma responses were successfully induced, we examined airway hyperresponsiveness, Th2 cytokines IL-4, and BALF. Our data showed that mice receiving TDI treatment exhibited increased airway hyperresponsiveness (Fig. 1A), higher IL-4 levels (Fig. 1B), and higher amounts of neutrophils and eosinophils in BALF than mice in the AOO group ($p < 0.05$) (Fig. 1C and D). Western blot analysis showed increased phosphorylation of Lrp6 (S1490) in the TDI-induced asthma group, which was dramatically suppressed by blockade of RAGE signaling via FPS-ZM1 (Fig. 1G). In addition, among the various wnt proteins, wnt3a and wnt10b showed elevated mRNA expression in the TDI group. No significant difference for these mRNAs were found in the RAGE inhibition group compared with the TDI group (Fig. 1E). Immunohistochemical staining showed aberrant distribution of β -catenin induced by TDI, mainly in airway epithelial cells, with considerable immunostaining in the cytoplasm and nucleus (Fig. 1F). Upregulation of β -catenin target genes, including VEGF, MMP9, and TGF β 1, was also observed in the TDI-induced asthma group (Fig. 1H), while treatment with the RAGE inhibitor suppressed the cytosolic and nuclear accumulation of β -catenin (Fig. 1E) as well as expression of β -catenin targeted genes (Fig. 1H).

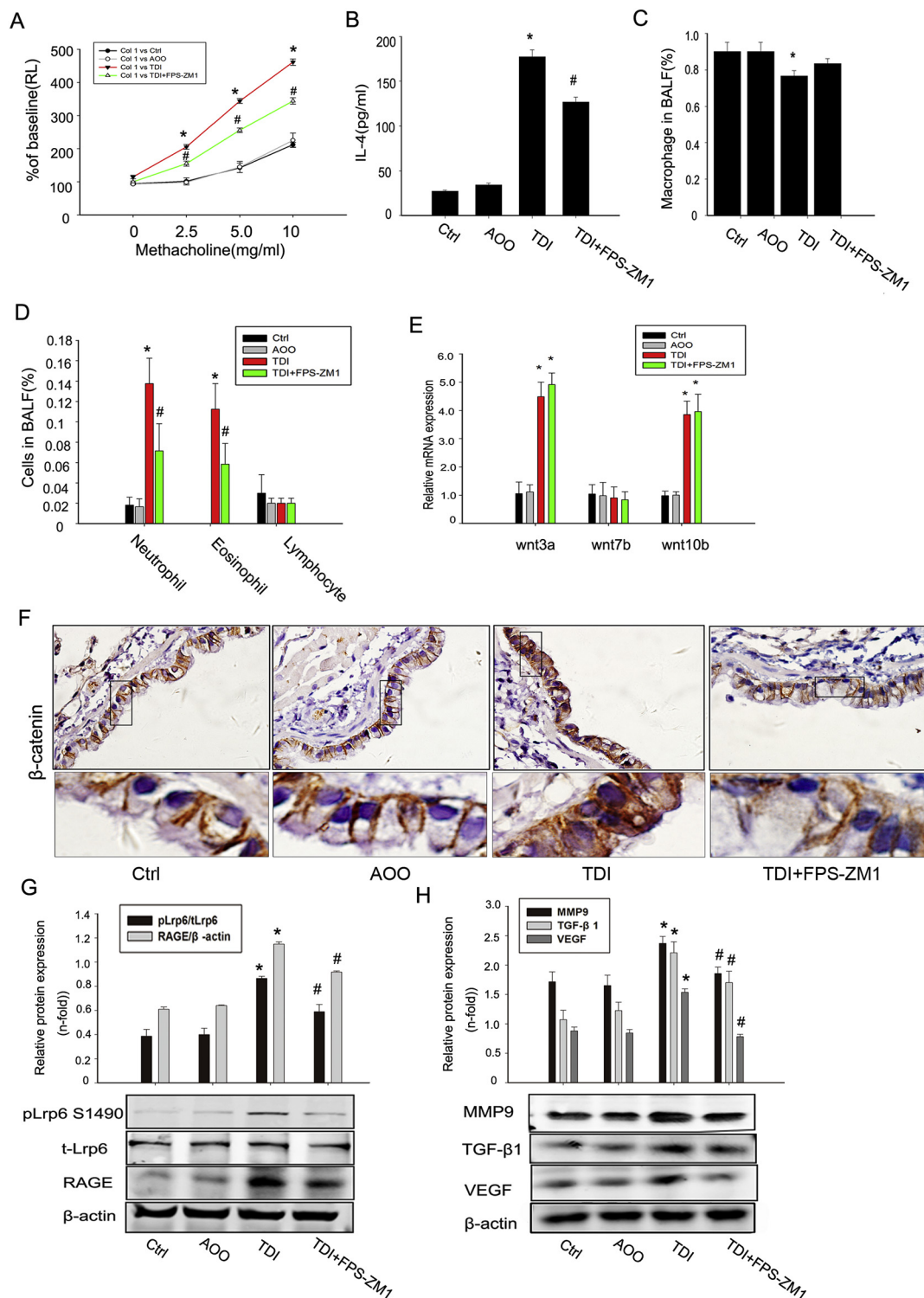


Fig. 1. The role of RAGE inhibition in canonical wnt/β-catenin signaling in a TDI-induced asthma model. (A) airway hyperresponsiveness to methacholine 24 h after the last challenge. (B) IL-4 in the supernatant of cultured lymphocytes was measured using ELISA. (C–D) Different cell counts of macrophages, neutrophils, eosinophils, and lymphocytes in BALF. (E) mRNA expression of wnt3a, wnt7b, and wnt10b in the whole lung. (F) Representative immunohistochemical staining of β-catenin in the bronchial regions. Original magnification was 400× (n = 6). (G) Western blot analysis of p-Lrp6, t-Lrp6, and RAGE in the whole lung. Representative immunoblots (bottom) and densitometric analyses (top) of protein levels normalized to β-actin levels were reported. (H) Western blot analysis of β-catenin target genes. Representative immunoblots (bottom) and densitometric analyses (top) of protein levels normalized to β-actin levels were reported. Data are expressed as mean ± SEMs (n = 6). *p < 0.05 compared with the AOO group; #p < 0.05 compared with the TDI group.

3.2. RAGE promotes TDI-HSA-induced β-catenin nuclear translocation in 16HBE cells

To further investigate the role of RAGE in mediating β-catenin

stabilization in airway epithelia, 16HBE cells were exposed to TDI-HSA conjugates. After treatment with various doses of TDI-HSA (40, 80, or 100 μg/ml) for 12 h, we observed a dose-dependent increase in RAGE expression induced by TDI-HSA (Fig. 2A). We then constructed stable

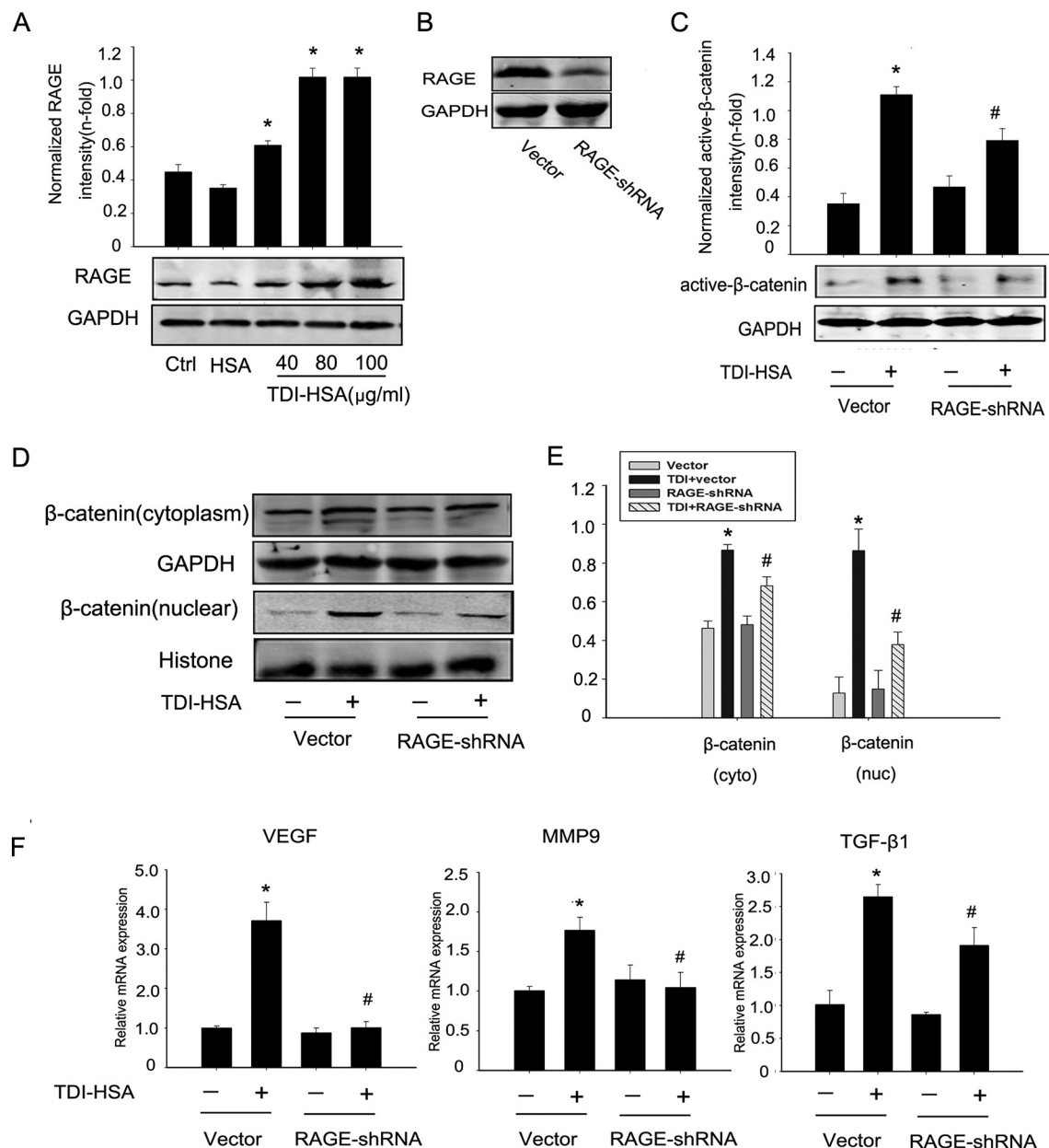


Fig. 2. The role of RAGE in TDI-induced β -catenin signal activation in 16HBE cells. (A) Western blot analysis of RAGE in 16HBE cells after various TDI-HSA treatments (40, 80, 100 μ g/ml), and representative immunoblots (bottom) and densitometric analysis (up) of the blots. (B) 16HBE cells were transfected with lentiviral-expressed RAGE shRNA and selected using puromycin. Successful transfection was confirmed by Western blot. (C) Western blot analysis of active- β -catenin in the different groups and densitometric analysis of the blots (up). (D) Western blot analysis of the cytoplasmic and nuclear translocation of β -catenin in the different groups after TDI-HSA treatment for 12 h, and densitometric analysis of the blots (E). (F) Quantification of the mRNA expression levels of β -catenin target genes. Data are mean $\pm$ SEMs of three independent experiments. * p < 0.05 compared with the control or vector group; # p < 0.05 compared with the TDI-HSA or TDI-HSA vector group.

RAGE knockdown 16HBE cell lines using lentiviral shRNA plasmids, which were verified by Western blot analysis (Fig. 2B). As expected, silencing of RAGE significantly blocked TDI-induced upregulation of active β -catenin (Fig. 2C) as well as the cytoplasmic and nuclear accumulation of β -catenin (Fig. 2D and E). In addition, using real-time PCR, we found that loss of RAGE blocked TDI-induced upregulated expression of the VEGF, MMP9, and TGF β 1 mRNAs (Fig. 2F), which are considered canonical genes downstream of β -catenin. These results suggested that RAGE mediates TDI-induced β -catenin signaling activation in airway epithelial cells.

3.3. Silencing of RAGE expression disrupts TDI-HSA-induced Lrp6 phosphorylation

Since activation of Lrp6 is known as a vital upstream event of β -catenin nuclear translocation in canonical wnt/ β -catenin signaling, we examined the activity of Lrp6 following stimulation with TDI-HSA in 16HBE cells. As expected, a marked increase in phosphorylation of Lrp6 at Ser1490 was observed, which peaked within 1–3 h following stimulation with TDI-HSA (Fig. 3A). Next, we determined whether RAGE signaling affected TDI-induced Lrp6 phosphorylation in vitro. RAGE-shRNA 16HBE cells were stimulated with 80 μ g/ml TDI-HSA for 3 h. In line with the effects on β -catenin, silencing of RAGE significantly inhibited the TDI-induced serine phosphorylation of Lrp6. Additionally,

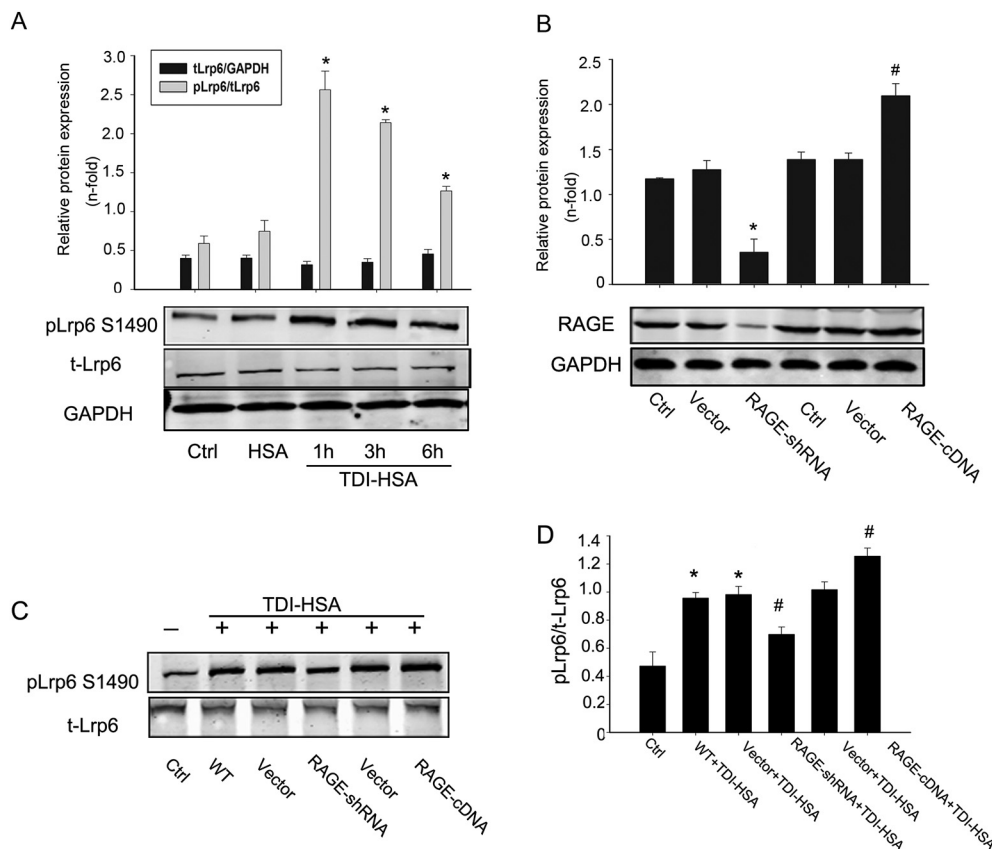


Fig. 3. The regulatory role of RAGE in mediating TDI-induced Lrp6 phosphorylation. (A) 16HBE cells were treated with 80 µg/ml TDI-HSA for various periods of time to detect expression of Lrp6 phosphorylation via Western blot analysis. Representative immunoblots (bottom) and densitometric analysis (up) of the blots. (B) 16HBE cells were transfected with lentiviral-expressed RAGE shRNA and RAGE cDNA and selected using puromycin. RAGE protein expression was confirmed by Western blot analysis in stably expressed RAGE shRNA and RAGE cDNA cells. Representative immunoblots (bottom) and densitometric analysis (up) of the blots. (C) Stable clones expressing the RAGE-shRNA and RAGE cDNA were treated with TDI-HSA (80 µg/ml) for 3 h and assessed for pLrp6 S1490 by Western blot. (D) Densitometric analysis of the blots. Data are mean $\pm$ SEMs of three independent experiments. * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the TDI-HSA + WT group.

RAGE overexpression in 16HBE cells exhibited considerably increased phosphorylation of Lrp6 compared with WT 16HBE cells (Fig. 3C). Together with our *in vivo* results, these observations suggested that RAGE modulates TDI-induced Lrp6 phosphorylation.

3.4. Involvement of the RAGE/ERK cascade in TDI-induced Lrp6 phosphorylation

To further explore the mechanism of Lrp6 phosphorylation, we determined whether wnts were involved. 16HBE cells were pre-incubated with the extracellular wnt-antagonist Dickkopf-1 (Dkk-1, 0.2 µg/ml), followed by stimulation with human recombinant wnt3a or TDI-HSA. As expected, treatment with exogenous wnt3a increased Lrp6 phosphorylation, while DKK-1 pretreatment inhibited this effect. In contrast, increased Lrp6 phosphorylation following stimulation by TDI-HSA was not inhibited by DKK-1 (Fig. 4A). These findings suggested that wnt secretion is not required for the observed TDI-induced Lrp6 activation. MAP kinases have been shown to mediate Lrp6 phosphorylation, and these kinases are also downstream of RAGE signaling. Therefore, we postulated that RAGE signaling might trigger MAPK kinase to mediate phosphorylation of Lrp6. Since we failed to observe the activation of JNK in 16HBE cells following TDI-HSA treatment in a previous study [20], we compared the activity of two other MAPK kinases (ERK and p38) in the control and RAGE-shRNA groups following stimulation via TDI-HSA. As shown in Fig. 4C, ERK and p38 were substantially activated by TDI-HSA. Additionally, knockdown of RAGE strongly decreased ERK phosphorylation, with weak changes in p38 phosphorylation. Next, we proceeded to test the role of ERK MAP kinase in mediating Lrp6 phosphorylation. 16HBE cells were pre-incubated with the ERK inhibitor (U0126) for 1 h and then treated with TDI-HSA. As can be seen in Fig. 4D, TDI-HSA-induced Lrp6 phosphorylation at S1490 was inhibited by U0126 in a dose-dependent manner. Therefore, these results suggested that the RAGE/ERK cascade

contributes to TDI-induced Lrp6 serine phosphorylation.

3.5. Silencing of Lrp6 inhibits TDI-induced β -catenin nuclear translocation

To identify the role of Lrp6 in mediating TDI-induced β -catenin stabilization, we knocked down Lrp6 via Lrp6 siRNA. At 48 h post-transfection, cells were lysed and successful transfection was determined by Western blot analysis (Fig. 5A). The expression of active β -catenin was examined in 16HBE cells treated with TDI-HSA. We found that Lrp6 knockdown effectively reversed the TDI-induced increase of active β -catenin (Fig. 5B). TDI-induced cytoplasmic and nuclear accumulation of β -catenin was also inhibited (Fig. 5C and E). In addition, qPCR analysis revealed that silencing of Lrp6 dramatically suppressed the TDI-induced upregulation of mRNA expression of β -catenin targeted genes, including VEGF, MMP9, and TGF- β 1 (Fig. 5F). These data suggested that Lrp6 likely played an important role in mediating TDI-induced β -catenin stabilization.

4. Discussion

In this study, we found that the wnt co-receptor Lrp6 was a potent positive regulator of β -catenin signaling in TDI-induced asthma models, both *in vivo* and *in vitro*. Additionally, for the first time, we demonstrated that RAGE could mediate phosphorylation of Lrp6, suggesting a functional cross talk between RAGE and the canonical wnt/ β -catenin signaling pathway involved in mediating β -catenin activation.

Previously, we found that RAGE was required for β -catenin stabilization in a TDI-induced murine asthma model [17]. However, the exact mechanism of β -catenin activation remained unclear. Aberrant activation of β -catenin has recently been proposed as a crucial regulator and therapeutic target for asthmatic airway remodeling [21], as demonstrated in airway smooth muscle cells and airway epithelium in both *in vivo* and *in vitro* models of asthma [22,23]. Aberrant activation

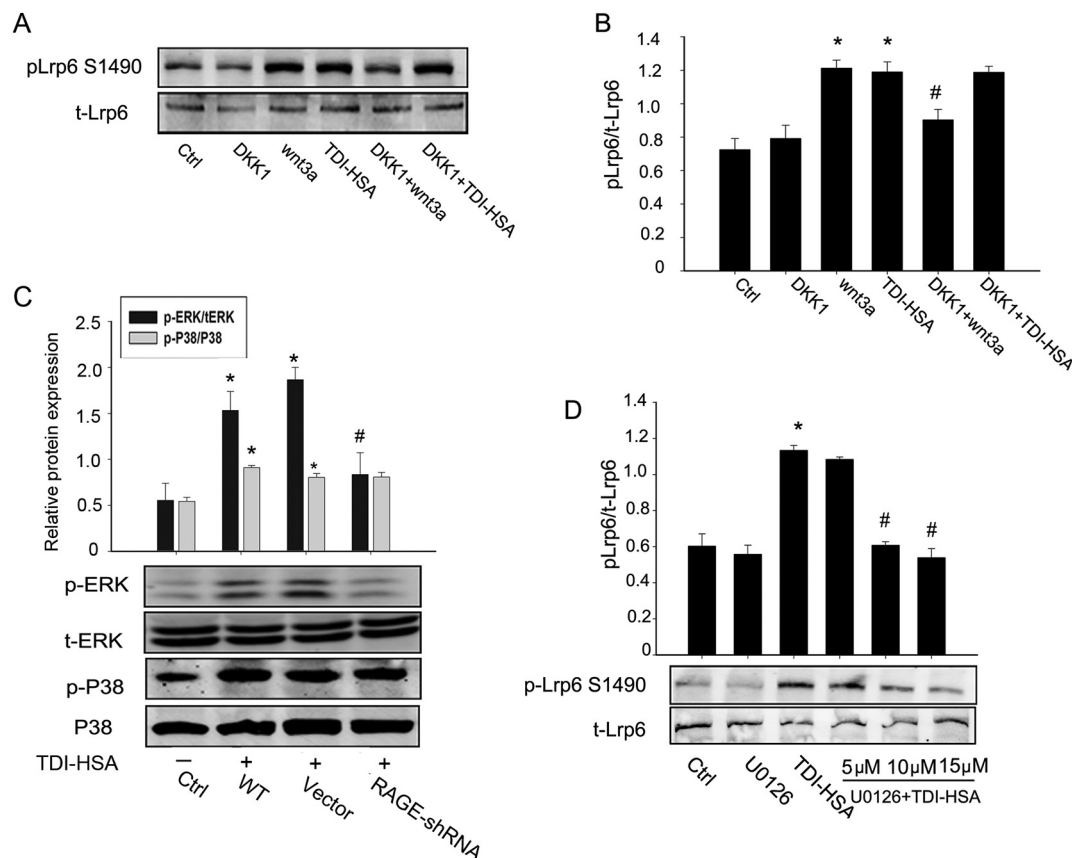


Fig. 4. RAGE phosphorylates Lrp6 partly through ERK1/2. (A) Western blot analysis for p-Lrp6 Ser-1490 and total Lrp6 in 16HBE cells that were pre-incubated with DKK1 followed by stimulation with wnt3a or TDI-HSA for 3 h. (B) Densitometric analysis of the blot. * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the wnt3a group. (C) Stable clones expressing RAGE-shRNA were treated with TDI-HSA for 3 h and MAPK-activated protein kinase activities were assessed. Representative immunoblots (bottom) and densitometric analysis (up) of the blots. (D) Cells were treated with the ERK inhibitor U0126 for 1 h prior to TDI-HSA treatment and analyzed for pLrp6 Ser-1490 by Western blot. Densitometric analysis of the blots is shown over the bands. Data are mean $\pm$ SEMs of three independent experiments. * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the TDI-HSA group.

of β -catenin can be mediated by both wnt-dependent and wnt-independent pathways in asthmatic airways [24]. Given that wnt signaling is still a well-established and important inducer of β -catenin activation, components of the canonical wnt/ β -catenin pathway were tested. In our study, several wnts (including wnt3a, wnt7b, and wnt10b), which represent the canonical wnt/ β -catenin pathway ligands [25–27], and the wnt co-receptor Lrp6 were assessed in our TDI-induced murine model to determine whether the canonical wnt/ β -catenin pathway was involved in β -catenin stabilization. The results showed that the expression of wnt3a and wnt10b were both upregulated in the TDI-induced murine model and this was accompanied by increased phosphorylation of Lrp6, indicating the involvement of the canonical wnt/ β -catenin pathway. Moreover, consistent with previous findings [17], denser immunostaining of β -catenin was observed in the cytoplasm and nucleus in airway epithelial cells of TDI-induced asthmatic mice, and this occurred along with significant upregulation of β -catenin targeted genes (VEGF, MMP9, and TGF β 1) [28–30], which further suggested activation of β -catenin signaling in the TDI-induced murine model.

As the first line of defense against allergens, the airway epithelium is considered an essential controller of inflammatory, immune, and regenerative response to allergens and environmental pollutants that contribute to asthma pathogenesis. These cells express pattern recognition receptors that detect environmental stimuli and secrete endogenous danger signals, thereby activating dendritic cells and bridging innate and adaptive immunity [31]. In our study, TDI challenge following dermal sensitization lead to aberrant distribution of β -catenin, which was most prominent in airway epithelial cells, while few changes

in the alveolar epithelia or smooth muscles were seen (data not shown). A recent study confirmed that β -catenin mediates the inflammatory cytokine expression induced by lipopolysaccharides in bronchial epithelial cells [32]. Similarly, we observed upregulated expression of VEGF, MMP9, and TGF- β 1 in human bronchial epithelial cells (16HBE) treated with TDI-HSA conjugates. Notably, these genes are downstream targets of β -catenin signaling and are also closely related to allergy induced airway inflammation and airway remodeling [33]. Numerous studies have confirmed that activation of β -catenin signaling can be mediated by Lrp6. Overexpression of the Lrp6 intracellular domain in cultured mammalian cells can accurately recapitulate Lrp6 signal transduction, decrease β -catenin phosphorylation, increase β -catenin levels, and upregulate wnt/ β -catenin target gene expression [34–36]. In our study, we found that knockdown of Lrp6 in 16HBE cells significantly suppressed the TDI-HSA-induced β -catenin nuclear accumulation and β -catenin target gene expression (i.e., VEGF, MMP9, and TGF- β 1), implying an essential role for Lrp6 in mediating TDI-induced β -catenin stabilization and airway inflammation.

To date, the effects of RAGE-ligand activation on wnt signaling have been explored in many cell types, and different effects have been demonstrated. It has been reported that AGE/RAGE promotes the calcification of human aortic smooth muscle cells via the wnt/ β -catenin axis [37]. While in osteoblasts, overexpression of RAGE reduces wnt/ β -catenin signaling and TCF/LEF transcriptional activity [38]. In our study, we found that treatment with the RAGE inhibitor FPS-ZM1 in vivo reduced cytoplasmic and nuclear levels of β -catenin in airway epithelial cells and, at the same time, downregulated protein expression of β -catenin target genes. In vitro, silencing of RAGE expression in 16HBE

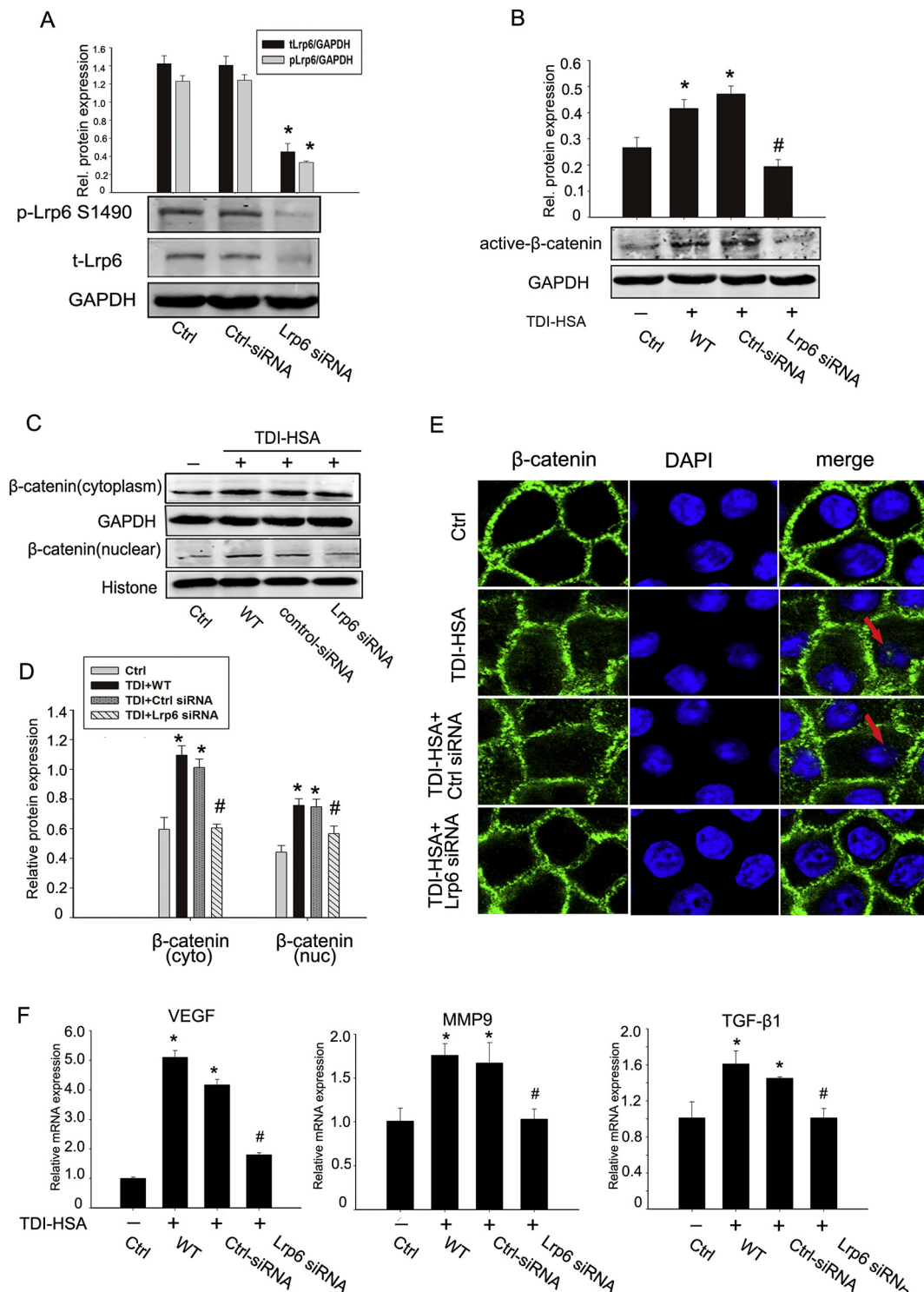


Fig. 5. The role of Lrp6 in mediating TDI-induced β -catenin stabilization. 16HBE cells were transfected with the Lrp6 siRNA and assessed at 48 h post-transfection. Successfully transfected cells were then exposed to 80 μ g/ml TDI-HSA for 12 h. (A) Successful Lrp6 siRNA transfection was determined by Western blot and densitometric analysis of the blots (up). (B) The effects of Lrp6 depletion on the expression of active- β -catenin. Representative immunoblots (bottom) and densitometric analysis (up) of the blots. (C) Western blot analysis of β -catenin cytoplasmic or nuclear translocation, and densitometric analysis of the blots (D). (E) Staining of β -catenin (green) was detected by fluorescence microscopy. (F) Quantification of the mRNA expression levels of β -catenin target genes following Lrp6 silencing. Data are mean $\pm$ SEMs of three independent experiments. * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the TDI-HSA group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells also disrupted TDI-induced β -catenin nuclear translocation and downregulated genes downstream of β -catenin. Of note, this RAGE inhibitor also effectively decreased the levels of phosphorylated Lrp6 in the lungs of TDI-exposed mice, while no significant differences in the

expression of several wnts were seen. This suggested that the RAGE axis may regulate Lrp6 activity in TDI-induced asthma. In 2016, Chen and colleagues reported that treatment with HMGB1, one of the RAGE ligands, induces EMT via a wnt/ β -catenin pathway and up regulates Lrp6

phosphorylation in human airway epithelial cells [18]. Similarly, we observed in our study that TDI-HSA exposure lead to β -catenin activation and increased phosphorylation of Lrp6 at Ser1490 in airway epithelia, and this could be inhibited by RAGE knockdown. Consistent with this, overexpression of RAGE in 16HBE cells resulted in increased serine phosphorylation of Lrp6, highlighting the regulatory role of RAGE during Lrp6 activation in airway epithelia. However, the mechanisms by which RAGE orchestrates Lrp6 phosphorylation still require further investigation.

In order to clarify the factors upstream of TDI-induced Lrp6 activation, we first examined whether the wnts could be involved. As wnt3a is the ligand of Lrp6 and Dkk1 can antagonize wnt-dependent Lrp6 phosphorylation by competitively binding to Lrp6 [26], we used wnt3a as a positive control for activation of the canonical wnt/ β -catenin signaling pathway in this study. We found that DKK1 effectively blocked the phosphorylation of Lrp6 induced by wnt3a, but it failed to suppress Lrp6 phosphorylation induced by TDI-HSA, indicating a wnt-independent pathway was likely involved in TDI-induced Lrp6 phosphorylation.

So far, multiple protein kinases have been shown to phosphorylate the PPPS/TP motifs of Lrp6 in both a wnt-dependent fashion, including GSK3, CK1, G protein-coupled receptor kinases (Grk5/6) [5], and in a wnt-independent fashion, including protein kinase A (PKA), PFTAIRE protein kinase1 (Pftk1), and mitogen-activated protein kinases (MAPKs) [14,15,39]. Previously, we found that ERK signaling is involved in both HMGB1- and TDI-induced impairment of airway epithelial barrier integrity [40,41]. Since several studies had identified MAPKs as downstream of the RAGE axis [42,43], we tested whether MAPKs were responsible for RAGE-mediated Lrp6 phosphorylation. Of interest, we observed increased activation of ERK1/2 and p38 MAPK (T180/Y182) after stimulation by TDI-HSA in 16HBE cells, while decreased levels of phosphor-ERK1/2 rather than phosphor-p38 were detected after RAGE knockdown, revealing that ERK may be implicated in RAGE-dependent responses in TDI-treated 16HBE cells. Subsequently, using the ERK inhibitor U0126, we found that TDI-induced Lrp6 phosphorylation was remarkably suppressed. Together, these results suggest that the RAGE/ERK cascade might be an underlining molecular mechanism involved in mediating TDI-stimulated Lrp6 phosphorylation.

5. Conclusion

In summary, we demonstrated that RAGE has a preeminent role in mediating TDI-induced β -catenin stabilization in a murine asthma model and in 16HBE cells. Involvement of the RAGE/ERK axis modulates Lrp6 phosphorylation, which contributes to β -catenin translocation in airway epithelial cells. Our data provide new insights into the mechanisms of TDI-induced β -catenin stabilization and suggest novel therapeutic targets for the treatment of TDI-induced asthma.

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Author contributions

C. S., X. J., Z. H. designed the experiment and wrote the manuscript. X. J. performed the experiments and analyzed the data. Z.W., L.Y and Y.L. helped with the experiments. Y.L., Y.C.X.G.H.G and D.H. helped with data analysis and revised the manuscript. All the authors agreed

that the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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