

The Elk1/MMP-9 Axis Regulates E-Cadherin and Occludin in Ventilator-Induced Lung Injury

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Research

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Abstract

Background: Ventilator-induced lung injury (VILI) is a common complication in the treatment of respiratory diseases with high morbidity and mortality. Matrix metalloprotein (MMP) 9 and Elk1 are involved in VILI, but the roles have not been fully elucidated. This study examined the mechanisms of the activation of MMP-9 and Elk1 regulating barrier function in VILI *in vitro* and *in vivo*.

Methods: For the *in vitro* study, MLE-12 cells were pre-treated with Elk1 siRNA or MMP-9 siRNA for 48 h prior to cyclic stretch at 20% for 4 h. For the *in vivo* study, C57BL/6 mice were pre-treated with Elk1 siRNA or MMP-9 siRNA for 72 h prior to 4h of mechanical ventilation. The expressions of Elk1, MMP-9, E-cadherin, and occludin were measured by Western blotting. The intracellular distribution of E-cadherin and occluding was shown by immunofluorescence. The degree of pulmonary edema and lung injury were evaluated by HE staining, lung injury scores, W/D weight ratio, total cell counts, and Evans blue dye.

Results: 20% cyclic stretch and high tidal volume could increase the expressions of Elk1 and MMP-9, decrease the E-cadherin and occludin level. Elk1 siRNA or MMP-9 siRNA could reverse the degradations of E-cadherin and occludin caused by cyclic stretch. Elk1 siRNA could decrease the MMP-9 level with or not 20% cyclic stretch and high tidal volume.

Conclusions: This study demonstrated that mechanical stretch could lead to the transcriptional activation of Elk1, enhance the expression of MMP-9 and mediate the loss of E-cadherin and occludin in VILI, thus indicating the therapeutic potential of Elk1 to treat VILI.

Background

Ventilator-induced lung injury (VILI) is mainly caused by the destruction of lung tissue, interstitial structures and alveolar membrane injury due to excessive alveolar dilatation or excessive intrapulmonary pressure[1–3]. VILI is primarily found in patients submitted to general anesthesia and/or intensive care units. The pathogenesis of VILI is based on increased permeability of the alveolar capillary membrane, which is associated with reduced or inactivated pulmonary surfactant and increased alveolar permeability and inflammation[4–6]. The clinical features of VILI include decreased lung compliance, pulmonary edema and impaired oxygenation, thus lead to acute respiratory distress syndrome (ARDS)[7, 8]. Therefore, more research is needed to better understand how to protect against lung injury caused by mechanical ventilation.

In this regard, tight junctions, which connect adjacent cells through tight protein particles, play an important role in alveolar permeability[9–11]. Our previous studies have found that high tidal volume mechanical ventilation can decrease the expressions of tight junctions E-cadherin and occludin, thus increasing alveolar permeability[12, 13]. The pathways regulating tight junctions have not been fully investigated. Therefore, we aimed to identify the activation mechanism of E-cadherin and occludin in VILI.

Matrix metalloprotein 9 (MMP-9) is thought to regulate the dynamic balance of extracellular matrix, neutrophil transmigration and barrier function[14–16]. MMP-9, which was shown to decrease the expressions of occludin and VE-cadherin, can also damage the blood-brain barrier integrity and permeability[17]. In VILI, increased MMP-9 in neutrophil transmigration was found to cause lung injury[18, 19]. However, it is yet unclear if MMP-9 regulated occludin and E-cadherin in VILI. Therefore, we aimed to identify the role of MMP-9 in VILI and assess if MMP-9 regulates E-cadherin and occludin.

ETS-domain containing protein (Elk1) is a transcription factor involved in regulating the expression of various genes involved in cellular growth, proliferation, apoptosis, tissue remodeling and angiogenesis[20]. Elk1 enhances its binding activity with DNA by its ETS domain, thus connecting the target gene promoter to regulate the expression and activity of downstream proteins[21]. Interestingly, the MMP-9 promoter region has ETS binding sites[22]. Therefore, we aimed to assess a possible association between Elk1 and MMP-9 in VILI.

Collectively, we hypothesized that high tidal volume mechanical ventilation could activate Elk1, thus upregulating the expression of MMP-9, which would inhibit E-cadherin and occludin in VILI.

Methods

Cell culture and treatment

Mouse lung epithelial cells (MLE-12) were purchased from American Type Culture Collection (Manassas, VA, USA). MLE-12 cells were seeded on collagen I-coated flexible-bottom BioFlex plates with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37 °C in a humidified atmosphere containing 5% CO₂. Elk1 siRNA (si-Elk1), MMP-9 siRNA (si-MMP-9) and Negative control siRNA (si-Nc) were constructed and synthesized by Gene Pharma Corporation, and transfected into 50% confluent MLE-12 cells with the Lipofectamine 3000 transfection reagent. After transfection for 48h, MLE-12 cells were treated with cyclic stretch. For the cyclic stretch, we used the FX-5000 T Flexercell Tension Plus system, and its parameters (VILI model *in vitro*) were set as follows: 20% stretch amplitude, a frequency of 30 cycles/min, a stretch-to-relaxation ratio of 1:1 applied in a cyclic manner, time of cyclic stretch for 2h or 4h.

Animal model and experimental protocol

All animal procedures were performed in accordance with the established guidelines, reviewed and approved by the Laboratory Animal Ethics Committee of Qianfoshan Hospital of Shandong University. C57BL/6 mice that weighed 20-25 g (6-8 weeks of age) were purchased from Vital River Laboratory and housed under specific pathogen-free conditions. For si-Elk1 or si-MMP-9 *in vivo* transfection, mice were injected by caudal vein with si-Elk1 (2.5µg/g) or si-MMP-9 (2.5µg/g), 10% glucose solution, DEPC water, and the Entranster™ In Vivo Transfection reagent (Engreen, 18668-11-1, Beijing, China) at the dosage of 200 µL according to the manufacturer's instructions. Animals were fasted for 24 h before experiments

and given free access to water. After 72 h of transfection, the mice were treated with mechanical ventilation.

The ventilation parameters (VILI model *in vivo*) were set as follows: tidal volumes of 20mL/kg, a respiratory rate of 40 times/min, PEEP of 0 cm H₂O, an inspiratory-to-expiratory (I/E) ratio of 1:2, a fraction of inspired oxygen of 21% and a time of mechanical ventilation of 2h or 4h.

All mice received the same standard diet during the experimental period. The mice were anesthetized via an intraperitoneal injection of pentobarbital and ketamine. Pancuronium was used to maintain muscle relaxation.

Bronchoalveolar lavage fluid (BALF)

After mechanical ventilation, the mice were treated with precooled saline (0.3 mL) into the lungs injected by tracheal intubation, and then pumped back after three seconds. The same operation was repeated three times. The number of cells was measured in the BALF.

Histopathological analysis

After mechanical ventilation, the mice were euthanized, and the lung injury scores were recorded. The upper lobe of the right lung was fixed in 4% paraformaldehyde for 72 h and embedded in paraffin. Tissue blocks were cut into 5- μ m slices, stained with hematoxylin for 5 min and eosin for 2 min (HE). The HE staining sections were observed under a light microscope at a magnification of 400 \times .

Lung wet/dry (W/D) weight ratio

After mechanical ventilation, the left lung was collected after heparin was injected through the postcava vein. The lung was weighed to determine the wet lung weight, then dried at 65 °C for 48 h and weighed again to determine the dry weight. The lung W/D ratio was calculated.

Evans blue dye extravasation

The Evans blue dye was injected by the tail vein 1h before mice were euthanized. The lungs were transferred, weighed, incubated with formamide (500 μ L) at 55 °C for 24 h to extract the dye. The formamide/Evans Blue mixture was centrifuged to pellet any remaining tissue fragments. The optical density was determined spectrophotometrically at 620 nm. The results were expressed as nanograms of dye per microgram of wet tissue.

Immunofluorescences

MLE-12 cells and lung tissue sections were permeabilized with immunostaining permeabilization buffer containing saponin for 5 min, then blocked with 5% BSA for 30 min at room temperature, and incubated with anti-E-cadherin (1:100, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-occludin (1:100, Abcam, Cambridge, MA, USA) antibodies diluted in 5% BSA overnight at 4 °C. The specimens were incubated with

green-fluorescent Alexa Fluor 488 donkey anti-rabbit IgG or red-fluorescent Alexa Fluor 594 rabbit anti-mouse IgG (Invitrogen, Grand Island, NY, USA) at room temperature for 1h after primary antibodies were washed off. The nuclei were stained with 4',6-diamidino-2-phenyl indole dihydrochloride (DAPI) for 5 min. A high-sensitivity laser confocal microscope was used to observe the changes of E-cadherin and occludin.

Western blotting and immunoprecipitation

After mechanical ventilation or cyclic stretch, the remaining right lung tissue or MLE-12 cells respectively were lysed on ice in a mixture of RIPA and PMSF (Beyotime, China). Equal amounts of protein were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, blocked in 5% nonfat milk for 2h at temperature, and incubated with primary antibodies at 4°C overnight. The primary antibodies were as follows: E-cadherin (1:500), occludin (1:1000), Elk1 (1:500, Abcam, Cambridge, MA, USA), MMP-9 (1:500, Santa Cruz Biotechnology, Dallas, TX, USA), and GAPDH (1:1000, Abcam, Cambridge, MA, USA). The membranes were incubated with goat anti-mouse or goat anti-rabbit secondary antibodies for 2 h at room temperature. The ECL SuperSignal reagent (Millipore, Billerica, CA, USA) was used to detect the protein bands by FluorChem E (ProteinSimple, CA, USA). The AlphaView software (ProteinSimple, CA, USA) was used to analyze the relative densities of the proteins. All experiments were performed in triplicates.

After treatment with cyclic stretch for 0h or 4h, MLE-12 cells were lysed in buffer with a protease cocktail for 30 min. The lysate was separated by centrifugation for 30 min. A small amount of supernatant was used for input, and the other part was precleared using isotype control IgG and protein A/G plus-agarose beads (20 µL) for 4 h, and then incubated with anti-Elk1 antibody and protein A/G plus-agarose beads (20 µL) at 4 °C overnight. The immunoprecipitated proteins were dissolved in 2× loading buffer for immunoblot analysis.

Statistical analysis

All data are expressed as mean ± SD. Significant differences were assessed using Student's t-tests or Tukey and least significance difference (LSD, L) with one-way analysis of variance (ANOVA), when appropriate. Statistical analysis was performed using the SPSS 26.0 statistics package for windows. A *P*-value <0.05 was considered statistically significant.

Results

***In vivo* and *in vitro* VILI models present the activation of Elk1 and MMP-9 and loss of E-cadherin and occludin**

In vivo, mice were treated with a high tidal volume (20mL/kg) for 0h, 2h, and 4h, while *in vitro*, MLE-12 cells were treated with a 20% cyclic stretch for 0h, 2h and 4h. The expressions of Elk1 and MMP-9 were increased, while E-cadherin and occludin decreased in a time-dependent manner after mechanical

ventilation or cyclic stretch (Figure 1A-E, Figure 2A-E). The immunofluorescence of lungs and MLE-12 cells showed that the intracellular distribution of E-cadherin and occludin decreased in a time-dependent manner (Figure 1F, Figure 2F).

Elk1 or MMP-9 knockdown regulated the loss of E-cadherin and occludin in VILI

In vitro, MLE-12 cells were pre-treated with si-Elk1 or si-MMP-9 and subjected to a 20% cyclic stretch for 4h. The expression of Elk1 was 80% lower in si-Elk1 cells, while there was a 70% decrease in si-MMP-9 cells when compared to normal cells (Figure 3A-D). Both si-Elk1 and si-MMP-9 abolished the down-regulation of E-cadherin and occludin induced by cyclic stretch (Figure 3E-L). The immunofluorescence study confirmed that si-Elk1 or si-MMP-9 ameliorated the intracellular distribution of E-cadherin and occludin caused by cyclic stretch (Figure 3M).

Elk1 was shown to regulate MMP-9 in VILI

Immunoprecipitation was used to detect the physical interaction of Elk1 and MMP-9 (Figure 4). Si-Elk1 or si-MMP-9 were used to investigate the relationship between Elk1 and MMP-9 *in vitro*. The expressions of Elk1 and MMP-9 were measured via western blotting. The expression of MMP-9 decreased when MLE-12 cells were treated with si-Elk1 and subjected to cyclic stretch, when compared to normal cells. The expression of Elk1 was not affected by MMP-9 downregulation (Figure 5).

Role of Elk1 and MMP-9 in regulating VILI in vivo

Mice were treated with si-Elk1 or si-MMP-9 and subjected to mechanical ventilation 4h. HE staining of lungs showed that edema, atelectasis, necrosis, inflammation, hemorrhage, and hyaline membrane formation were aggravated by mechanical ventilation, compared to healthy mice. These phenomena were reversed by si-Elk1 or si-MMP-9. Total cell counts, lung injury scores, W/D weight ratio, and Evans blue dye extravasation showed that high tidal mechanical ventilation could cause lung injury, while si-Elk1 or si-MMP-9 ameliorated the lung injury (Figure 6).

The expressions of E-cadherin, occludin, Elk1, and MMP-9 were measured by western blotting. The expressions of E-cadherin and occludin increased in mice treated with si-Elk1 or si-MMP-9 subjected to high tidal volume mechanical ventilation. Both Elk1 and MMP-9 were activated in mice subjected to high tidal volume mechanical ventilation. Meanwhile, the expression of MMP-9 decreased when mice were treated with si-Elk1. In contrast, the levels of Elk1 were similar in mice treated with or without si-MMP-9. Immunofluorescence analysis evidenced that intracellular E-cadherin and occludin were observed in mice treated with si-Elk1 or si-MMP-9 and high tidal volume mechanical ventilation (Figure 7).

Discussion

The current study demonstrates the roles of Elk1 and MMP-9 in regulating tight junctions on VILI models *in vivo* and *in vitro*. The major findings of this study are: (1) High tidal volume and 20% cyclic stretch caused VILI *in vivo* and *in vitro*, respectively; (2) Elk1 and MMP-9 contributed to tight junctions on VILI

both *in vivo* and *in vitro*; (3) downregulation of Elk1 or MMP-9 alleviated VILI and (4) Elk1 could regulate the expression of MMP-9.

Mechanical ventilation can cause ARDS if not used properly in anesthesia and intensive care units[23]. Thus, it is important to study the underlying mechanisms of VILI. The VILI model in mice and MLE-12 cells previously reported by our team was used to simulate ventilator-induced lung injury seen in the clinic[24]. This study demonstrated that high tidal volume mechanical ventilation caused lung injury as assessed by lung injury scores, W/D weight ratio, Evans blue dye extravasation, and HE staining. The mechanisms herein assessed include activation of inflammatory responses; barrier function damage and changes in sodium, potassium, and calcium plasma channels. This study sought to investigate the mechanisms regulating barrier function.

The alveolar epithelial barrier plays an important role in lung barrier function since it maintains lung tissue homeostasis, participates in pathogen defense, and regulates immune responses[25, 26]. Tight junctions are an integral part of the lung barrier. Occludin and E-cadherin are typical proteins of tight junctions. Thus, we used occludin and E-cadherin as indicatives of the alveolar epithelial barrier. In this study, we showed that high tidal volume mechanical ventilation and 20% cyclic stretch could decrease the expressions of E-cadherin and occluding, thus damaging the alveolar epithelial barrier function in a time-dependent manner. These findings are consistent with previous studies. However, underlying mechanisms should be further investigated.

The main function of MMP-9 is to damage and reshape the dynamic balance of the extracellular matrix[14]. Several recent studies have found that MMP-9 is involved in the expressions of E-cadherin or occludin in cerebral ischemia-reperfusion injury, acute lung injury, and acute kidney injury[27-29]. Thus, we investigated if MMP-9 could regulate tight junctions in VILI. We have demonstrated that MMP-9 could be activated to decrease E-cadherin and occludin in VILI both *in vivo* and *in vitro*. MMP-9 knockdown could reverse the expressions of E-cadherin and occludin to protect the lungs in VILI. Our findings indicate that mechanical stretch could activate MMP-9 to break the alveolar barrier integrity, thus leading to the loss of E-cadherin and occludin. The inhibition of MMP-9 could reverse the above phenomena.

Many recent studies showed that Elk1 plays an important role to regulate tight junctions in acute lung injury and intestinal epithelial barrier dysfunction[30, 31]. Our current findings suggest that Elk1 could be activated in VILI since Elk1 knockdown could increase the expressions of E-cadherin and occludin. Both MMP-9 and Elk1 participated in VILI, and the relationship between these two proteins was still unclear. Elk1 enhanced its binding activity with DNA through its ETS domain, while the MMP-9 promoter region has ETS binding sites. We speculated that MMP-9 and Elk1 interact. Our findings showed that mechanical stretch could increase the physical interaction of MMP-9 and Elk1. We used si-MMP-9 and si-Elk1 to confirm that Elk1 levels were not affected in si-MMP-9 cells subjected to cyclic stretch. However, si-Elk1 could knockdown the expression of Elk1 and MMP-9 at the same time. Therefore, our findings indicate that Elk1 was upstream of MMP-9 in our VILI model.

Conclusions

In conclusion, this study clarifies underlying mechanisms that mediate the degradation of E-cadherin and occludin in VILI. Mechanical stretch can lead to the transcriptional activation of Elk1, enhance the expression of MMP-9 and mediate the loss of E-cadherin and occludin in VILI, thus indicating the therapeutic potential of Elk1 to treat VILI.

Abbreviations

VILI, Ventilator-induced lung injury; MMP-9, Matrix metalloprotein 9; siRNA, Small interfering RNA; ARDS, Acute respiratory distress syndrome; HE, Hematoxylin Eosin; BALF, Bronchoalveolar lavage fluid; DAPI, 4',6-diamidino-2-phenylindole; BSA, Albumin from bovine serum; RIPA, Radio immunoprecipitation assay; PMSF: Phenylmethanesulfonyl fluoride; ANOVA, one-way analysis of variance.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TZ, JY, MZ, CG, FY, HW, and YW carried out the experiments, participated in the molecular biology studies. TZ carried out the immunoassays, participated in performed the statistical analysis and drafted the manuscript. YW participated in the design and conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

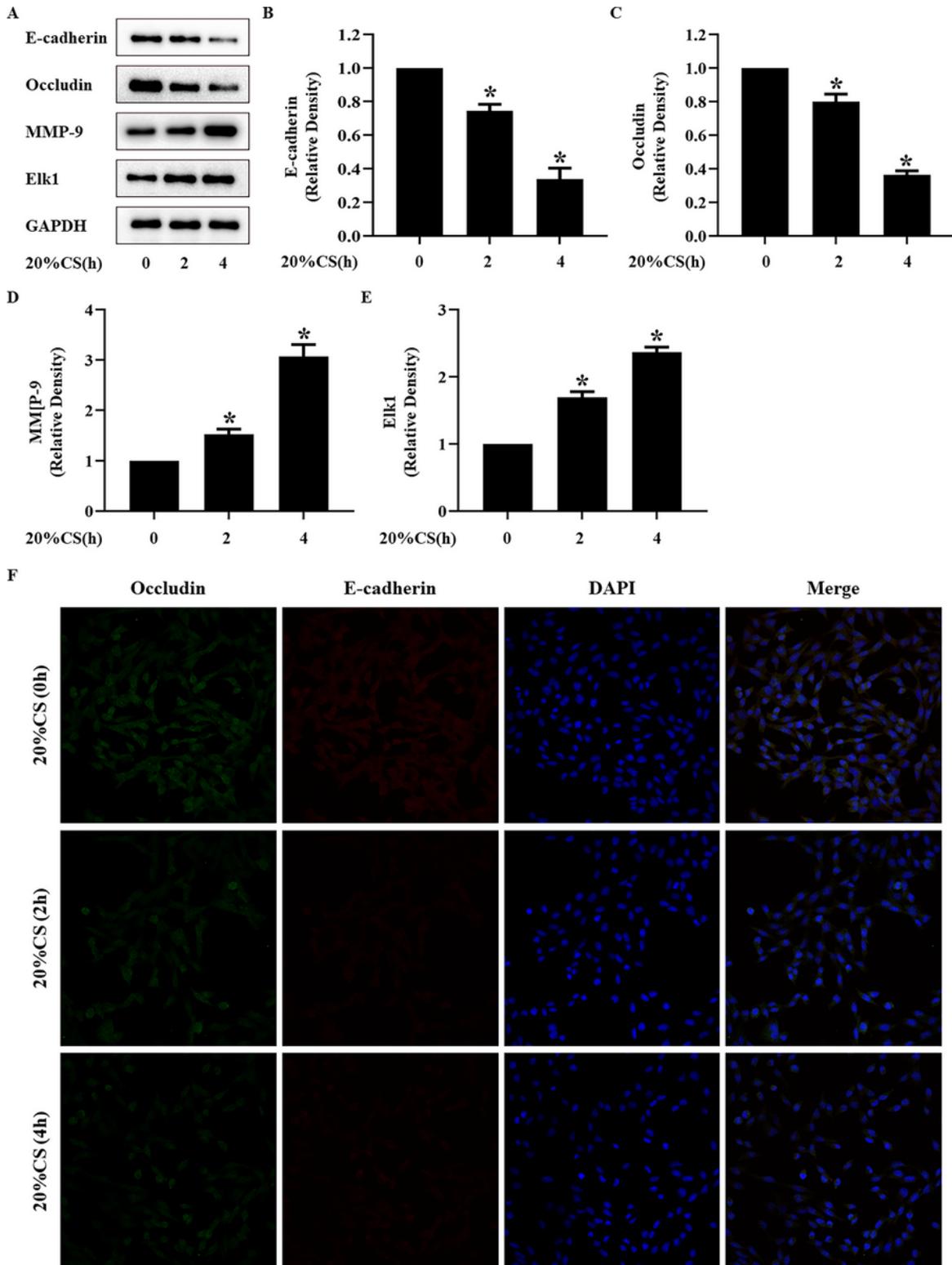


Figure 1

20% cyclic stretch induced Elk1 and MMP-9 activation and Occludin and E-cadherin degradation in MLE-12 cells. MLE-12 cells were treated with 20% cyclic stretch for 0h, 2h, and 4h. A: Representative Western blotting of E-cadherin, Occludin, Elk1, and MMP-9. B, C, D, E: The density of the proteins at 0h was used as a standard. * $P < 0.05$, vs. the CS 0h group. F: Immunofluorescence was used to detect the distribution of

Occludin (green) and E-cadherin (red), and the nuclei were stained with DAPI (blue). All experiments were repeated at three times.

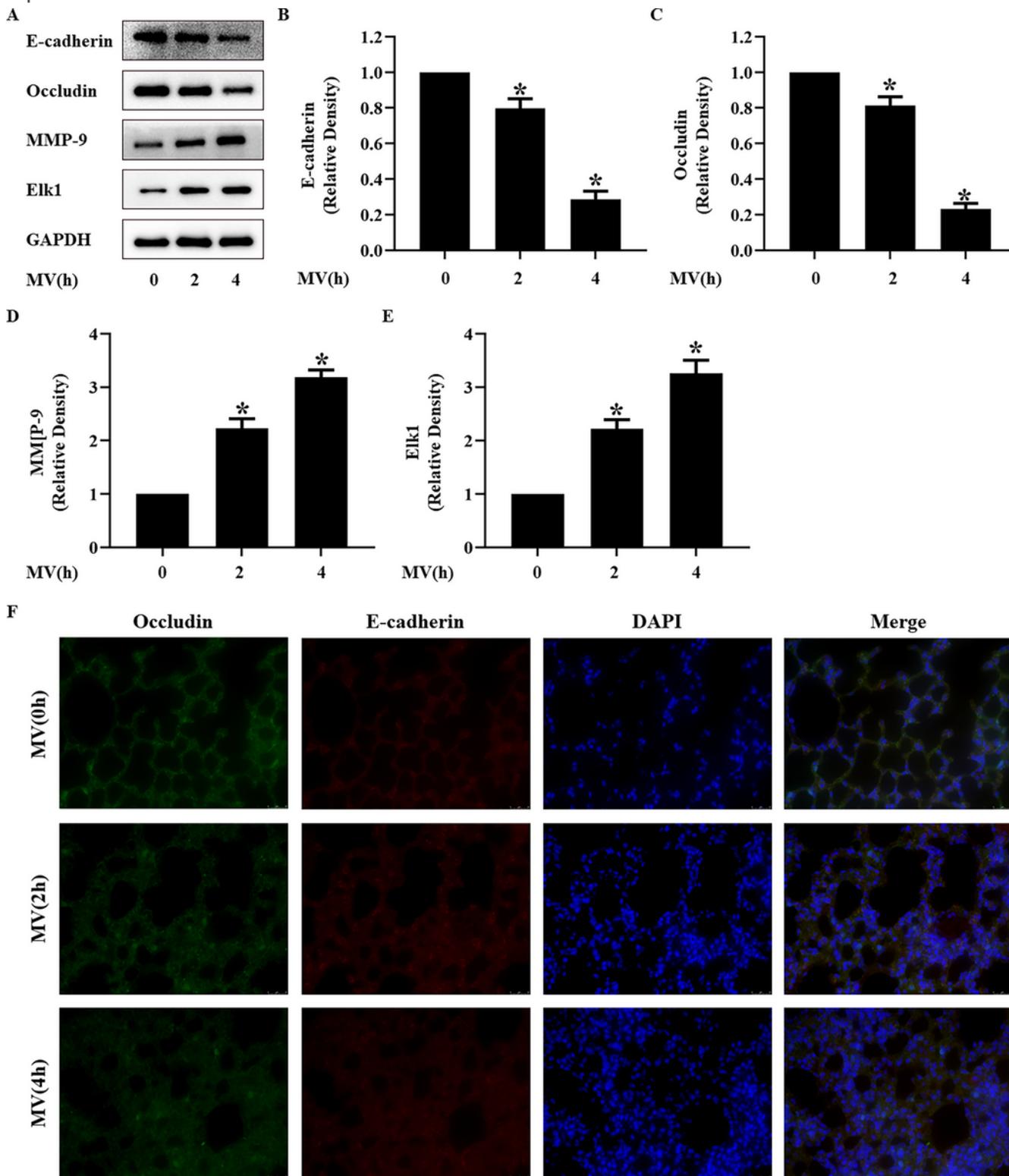


Figure 2

High tidal volume mechanical ventilation induced Elk1 and MMP-9 activation and Occludin and E-cadherin degradation in C57BL/6 mice C57BL/6 mice were treated with high tidal volume (20mL/kg) for 0h, 2h, and 4h. A: Representative Western blotting of E-cadherin, Occludin, Elk1, and MMP-9. B, C, D, E:

The density of the proteins at 0h was used as a standard. *P<0.05, vs. the MV 0h group. F: Immunofluorescence was used to detected the distribution of Occludin (green) and E-cadherin (red), and the nuclei were stained with DAPI (blue). All experiments were repeated at five times.

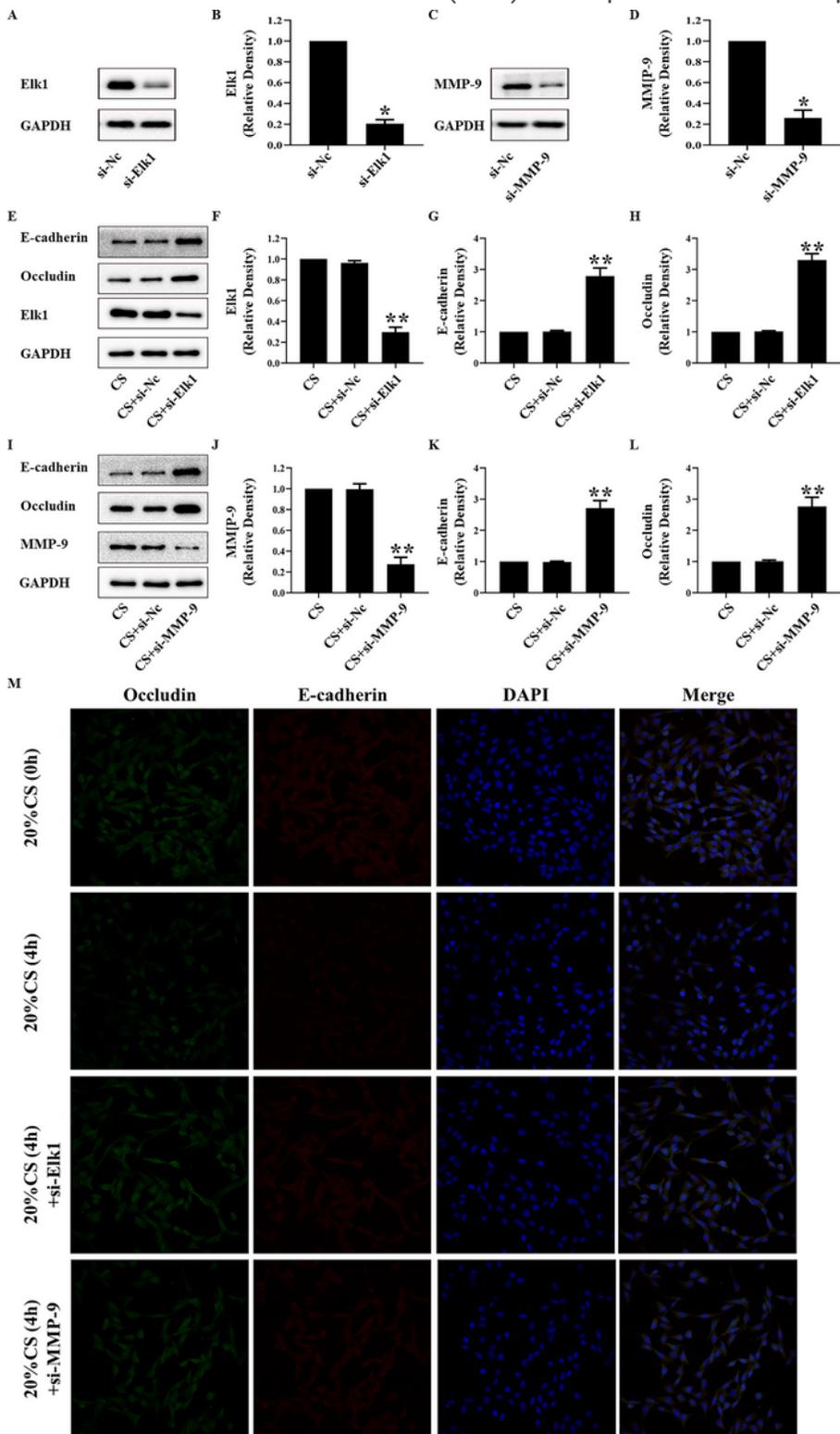


Figure 3

MMP-9 and Elk1 mediated the degradation of Occludin and E-cadherin after 20% cyclic stretch A and C: Representative Western blotting of Elk1 and MMP-9 in MLE-12 cells treated with si-Elk1 or si-MMP-9. B

and D: The density of the proteins at si-Nc was used as a standard. *P<0.05, vs. the si-Nc group. E and I: Representative Western blotting of Elk1, MMP-9, E-cadherin, and Occludin in MLE-12 cells treated with si-Elk1 or si-MMP-9 and 20% cyclic stretch for 4h. F, G, H, J, K, L: The density of the proteins at CS was used as a standard. **P<0.05, vs. the CS group. M: Immunofluorescence was used to detected the distribution of Occludin (green) and E-cadherin (red), and the nuclei were stained with DAPI (blue) in MLE-12 cells treated with si-Elk1 or si-MMP-9 and 20% cyclic stretch for 4h. All experiments were repeated at three times.

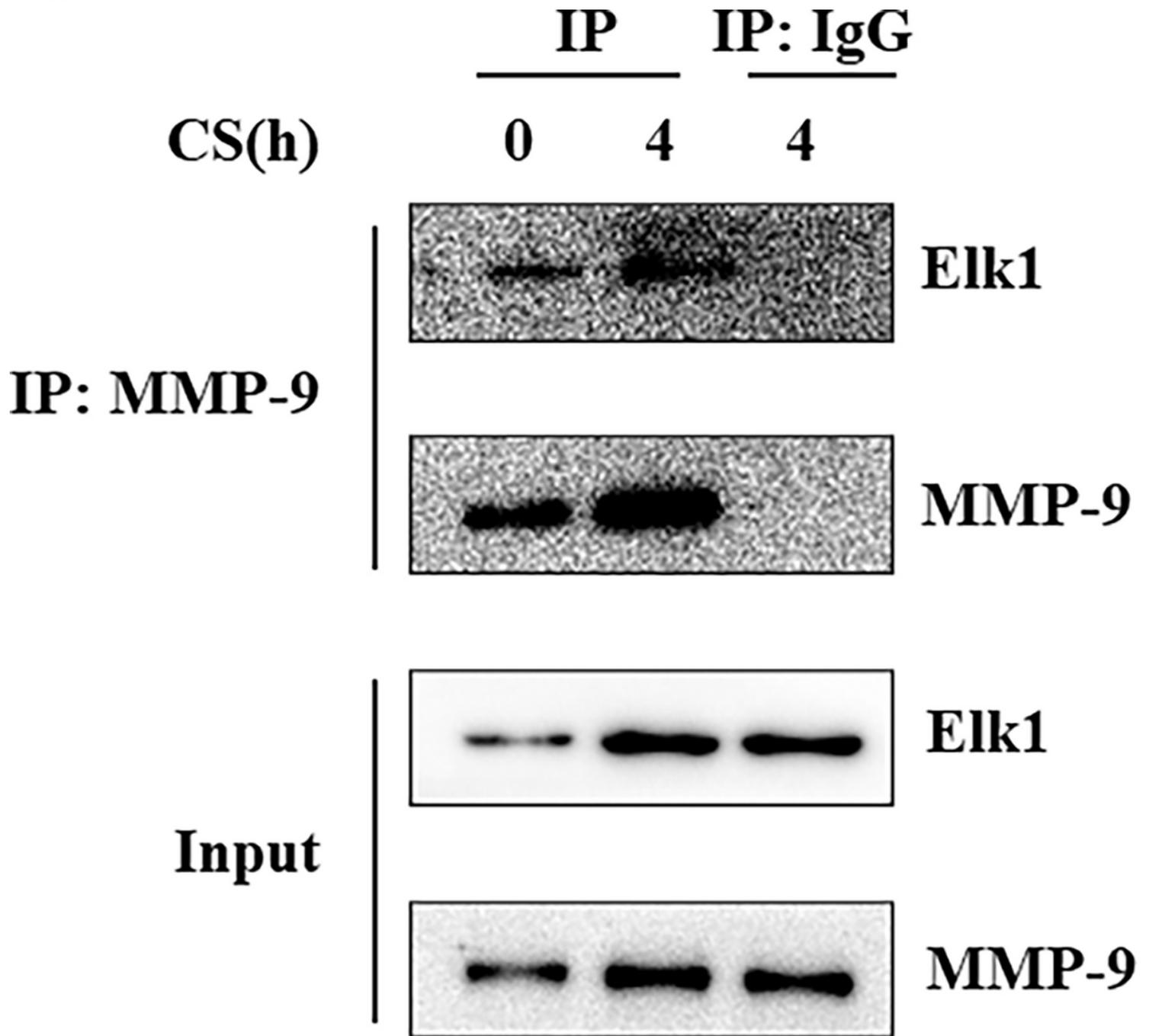


Figure 4

The combination of Elk1 and MMP-9 in MLE-12 cells The combination of Elk1 and MMP-9 was detected by immunoprecipitation after 20% cyclic stretch for 0h, 4h.

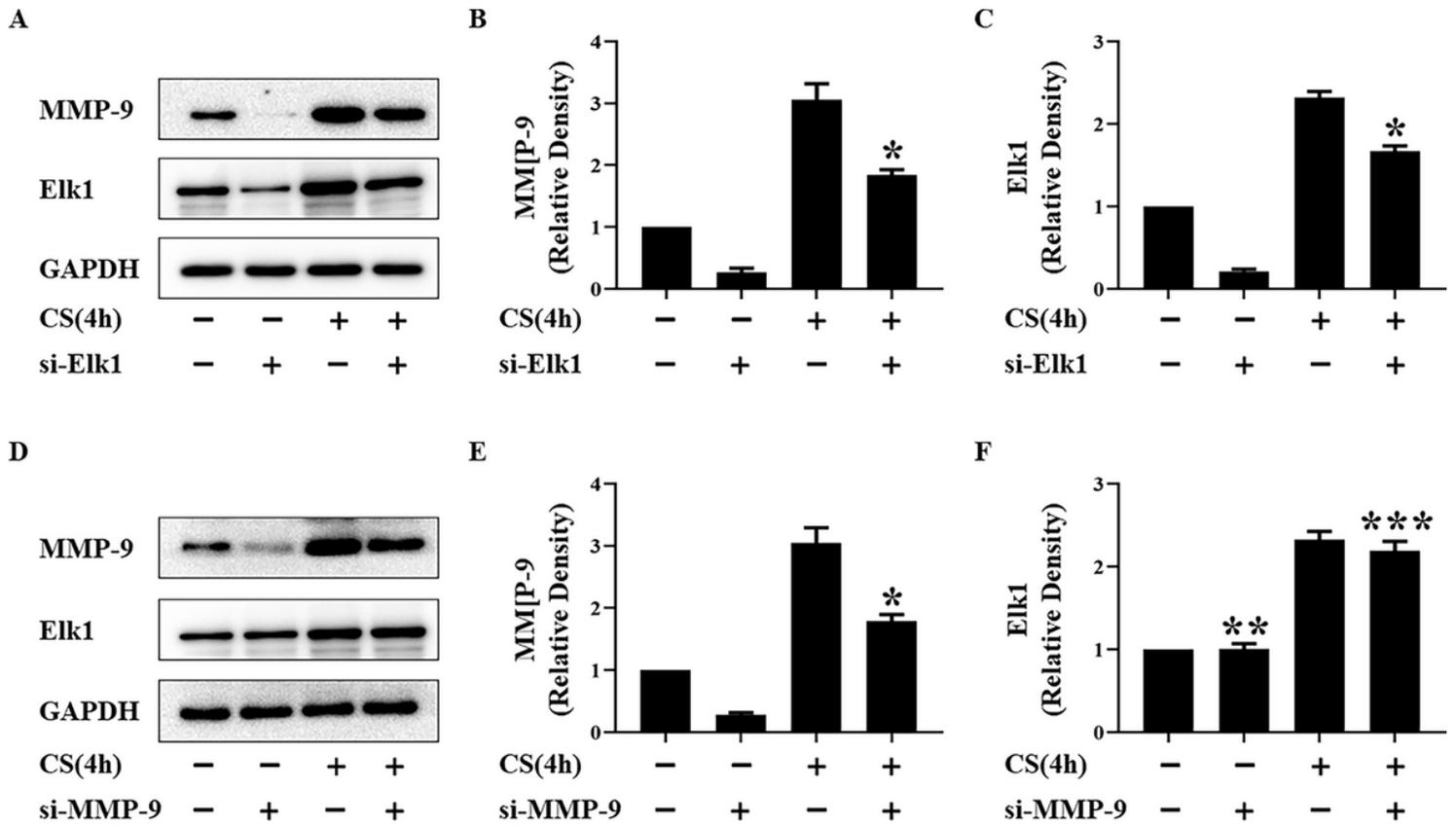


Figure 5

Expressions of Elk1 and MMP-9 in MLE-12 cells treated with or without si-Elk1 or si-MMP-9 or 20% cyclic stretch A and D: Representative Western blotting of Elk1 and MMP-9 in MLE-12 cells. B, C, E, F: The density of the proteins at the control group was used as a standard. * $P < 0.05$, vs. the CS group. ** $P > 0.05$, vs. the control group. *** $P > 0.05$, vs. the CS group.

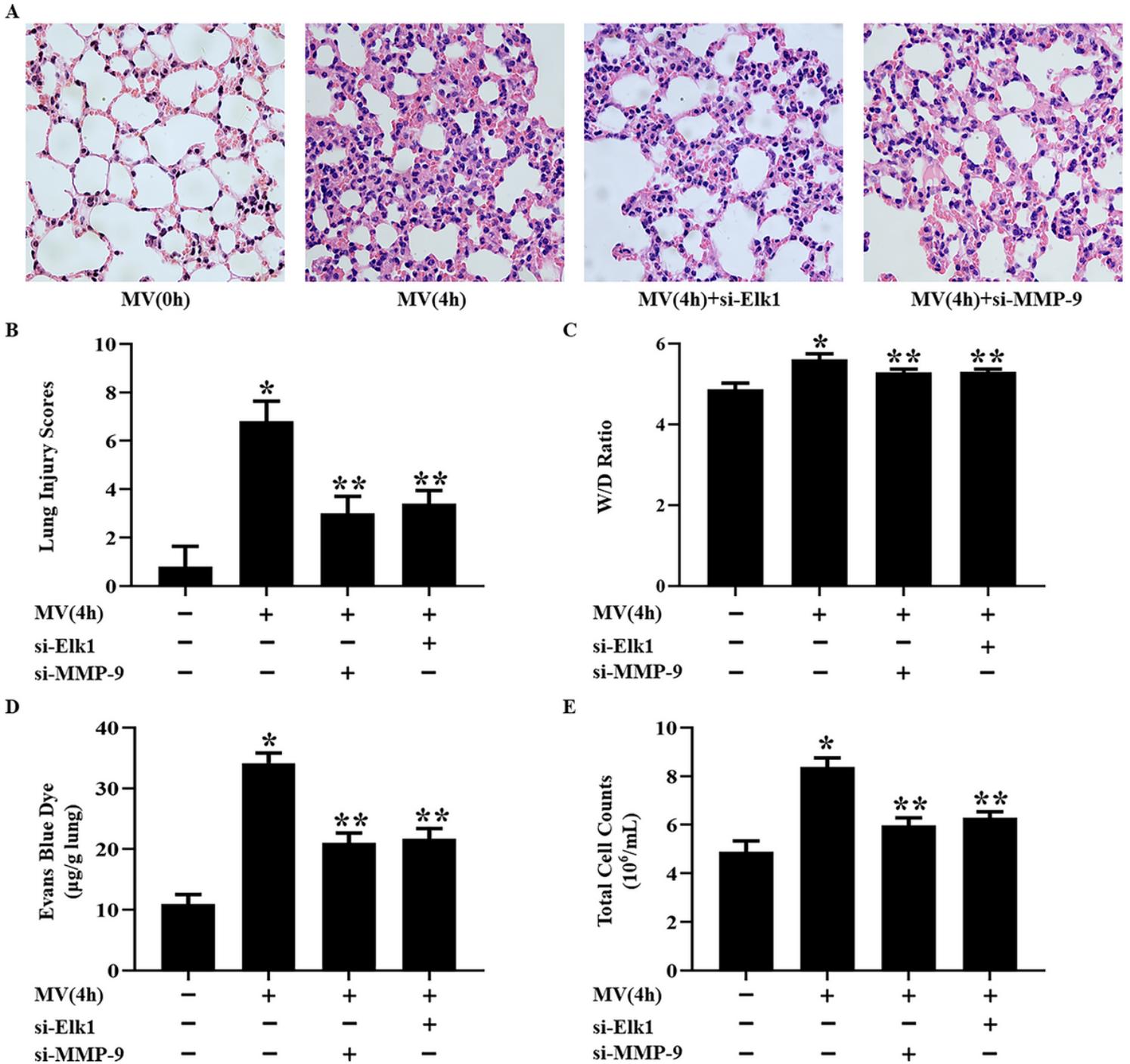


Figure 6

si-Elk1 or MMP-9 ameliorated the pulmonary edema and lung injury in VILI A: The pathological changes of lung tissues were determined by HE staining (magnification 400×). B: lung injury scores, C: W/D weight ratio, D: Evans blue dye, E: Total cell counts were used to evaluate the degree of pulmonary edema and lung injury in different group. * $P < 0.05$, vs. the MV(0h) group. ** $P < 0.05$, vs. the MV(4h) group. All experiments were repeated at five times.

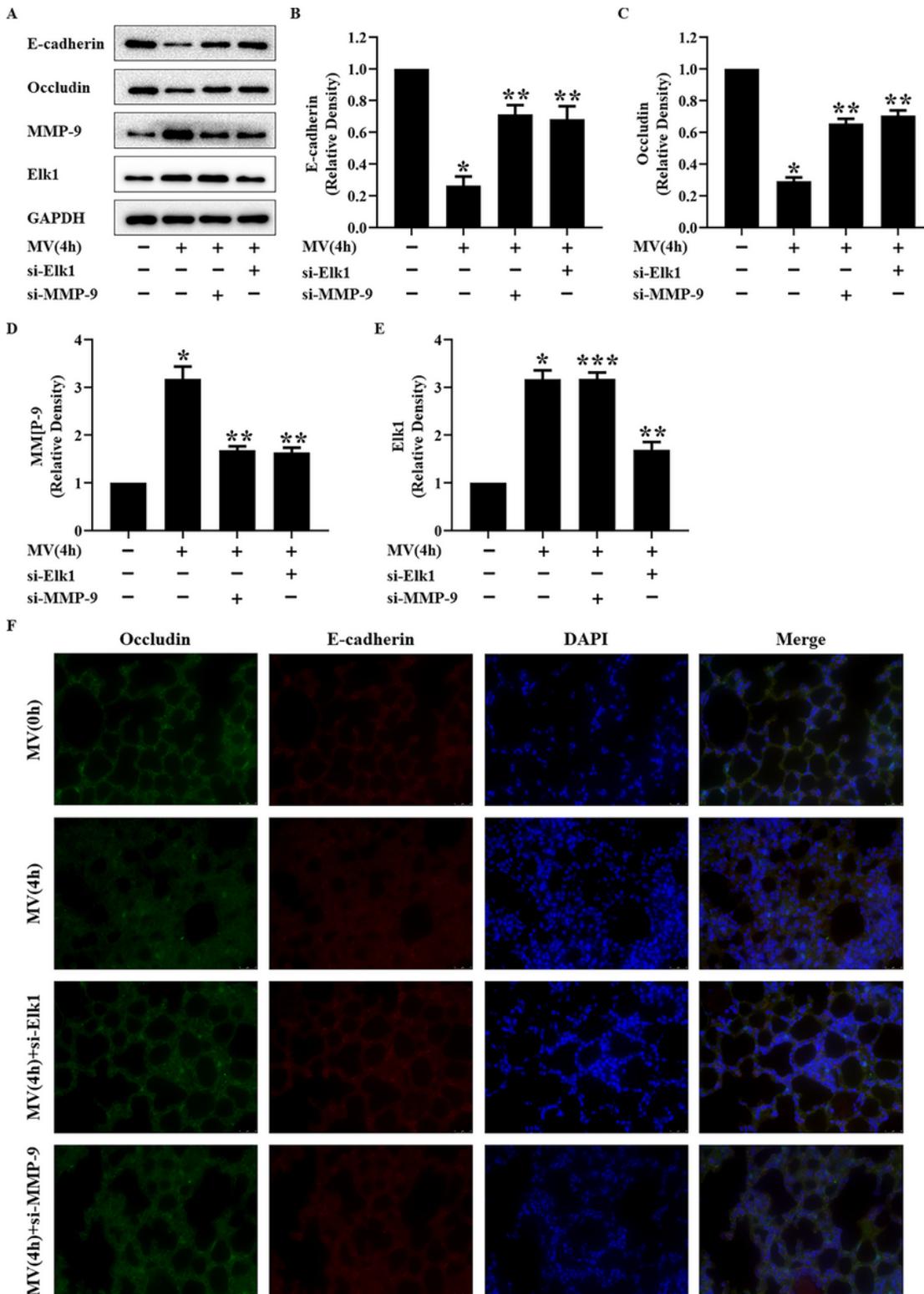


Figure 7

si-Elk1 or MMP-9 ameliorated the expressions of E-cadherin, occludin, Elk1, and MMP-9 A: Representative Western blotting of E-cadherin, occludin, Elk1 and MMP-9 in MLE-12 cells. B, C, D, E: The density of the proteins at the control group was used as a standard. * $P < 0.05$, vs. the control group. ** $P < 0.05$, vs. the MV(4h) group. *** $P > 0.05$, vs. the MV(4h) group. F: Immunofluorescence was used to detected the distribution of Occludin (green) and E-cadherin (red), and the nuclei were stained with DAPI (blue) in

C57BL/6 treated with si-Elk1 or si-MMP-9 and high tidal volume for 4h. All experiments were repeated at five times.