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MSCs reduce airway remodeling in the lungs of asthmatic rats through the Wnt/β-catenin signaling pathway

J. SONG[1], X.-M. ZHU[2], Q.-Y. WEI[2]

[1]Respiratory Medicine Doctoral Student, China Medical University, Shenyang, China
[2]Department of Allergy, General Hospital of Northern Theatre Command, Shenyang, China

Abstract. – OBJECTIVE: Asthma is a chronic pulmonary inflammatory disease characterized by excessive infiltration of leukocytes into the respiratory tract. We explored the underlying mechanisms of mesenchymal stem cells (MSCs) in the treatment of allergic asthma using a rat model.

MATERIALS AND METHODS: The rats were sensitized with ovalbumin (OVA) and an aluminium hydroxide emulsion, which were injected intraperitoneally, and then the sensitized rats were challenged with aerosolized OVA. Before the allergen challenge, the model rats were injected with MSCs and MSC-derived exosomes. At the same time, 2 out of the 6 groups of rats were injected with BML-284, a Wnt agonist. The degree of airway inflammation was determined by bronchoalveolar lavage fluid (BALF) and haematoxylin and eosin (H&E) staining; the degree of airway remodelling was assessed by Masson staining; Western blotting (WB) and real-time polymerase chain reaction (PCR) were performed to evaluate Wnt/β-catenin signalling pathway-related factors and the expression of epithelial-mesenchymal transition (EMT)-related proteins in lung tissues.

RESULTS: We showed that among the rats that were sensitized and challenged with OVA, the injection of MSCs and MSC-derived exosomes significantly reduced the total number of cells and the number of immune cells in BALF, proliferation of goblet cells and collagen deposition. Moreover, the number of BALF cells and collagen deposition increased significantly after the injection of BML-284. WB and real-time PCR showed that MSCs and MSC-derived exosomes significantly inhibited airway remodelling and EMT by restricting the Wnt/β-catenin signalling pathway, while additional injection of BML-284 suppressed the effects of MSCs and their exosomes, increased the EMT of the airway epithelium and exacerbated airway remodelling.

CONCLUSIONS: MSCs inhibit chronic allergic inflammation of the airway and reduce airway remodelling and EMT of the airway epithelium in the lungs of asthmatic rats. This process is partly attributed to the inhibition of the Wnt/β-catenin signalling pathway by MSC-derived exosomes.

Key Words:
Asthma, Mesenchymal stem cells, Exosomes, Wnt/β-catenin signalling pathway.

Abbreviations

MSCs: mesenchymal stem cells; OVA: ovalbumin; BALF: bronchoalveolar lavage fluid; H&E staining: hematoxylin and eosin staining; WB: Western blot; PCR: polymerase chain reaction; EMT: epithelium-mesenchymal transition; Th1 cells: T helper 1 cells; Th2 cells: T helper 2 cells; TGF-β: transforming growth factor-β; EGF: epidermal growth factor; FGF: fibroblast growth factor; HGF: hepatocyte growth factor; IL-6: interleukin-6; Axin: Axis inhibition protein; Smad: Mothers against decapentaplegic homolog; PI3K: Phosphatidylinositol 3 kinase; APC: Anaphase promoting complex; GSK-3 β: Glycogen Synthase Kinase 3 Beta; LRP: low-density lipoprotein receptor-related protein; ERK: Extracellular Signal Regulated Kinase; JNK: Jun N terminal kinase; c-Myc: Cellular myelocytomatosis oncogene; ECM: Extracellular matrix; MMPs: matrix metalloproteinases; TIMPs: tissue inhibitors of matrix metalloproteinase; GVHD: Graft-versus-host disease; MHC II: Major histocompatibility complex II; Tregs: regulatory T cells; IL-10: interleukin-10; HLA-G5: human leukocyte antigen G5; TCF: Transcription factor; SD rats: Sprague Dawley rats; PBS: Phosphate-buffered saline; FBS: fetal bovine serum; DMEM/F12: Dulbecco's Modified Eagle's Medium/F12; EDTA: ethylenediaminetetraacetic acid; P3: Passage 3; PE: Phycoerythrin; FITC: Fluorescein Isothiocyanate; DMEM-LG: Dulbecco's Modified Eagle's Medium-low glucose; PFA: Paraformaldehyde; DMEM-HG: Dulbecco's Modified Eagle's Medium-high glucose; DAB: Diaminobenzidine; BCA: bicinchoninic acid; RIPA: Radio Immunoprecipitation Assay; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride; HRP: Horseradish Peroxidase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NCBI: National Center for Biotechnology Information; Ct: threshold cycle; ANOVA: Analysis of Variance; TEM: transmission electron microscopy; IL-4: interleukin-4; IL-5: interleukin-5; IL-13: interleukin-13.

Corresponding Author: Qingyu Wei, Ph.D; e-mail: rxliu@cmu.edu.cn

Introduction

Asthma is a disease associated with disordered autoimmunity and it increasingly occurs worldwide. There are 15-20 million asthma patients in China, and the situation has worsened in recent years due to reasons including population and environmental issues[1]. Asthma is a chronic inflammatory reaction in which T cells, monocytes, mast cells, eosinophils, and other cells and their secreted components orchestrate a complicated pathogenic mechanism. The main feature of asthma is that the airway epithelium is infiltrated by inflammatory cells, resulting in epithelial-mesenchymal transition (EMT); airway epithelial cells undergoing the EMT and fibroblasts secrete a large amount of collagen. In addition, the inflammatory environment also causes hypertrophy and proliferation of airway smooth muscle. The excessive collagen and changes in airway smooth muscle further cause airway remodelling, which is one of the core pathological changes in asthma[2]. Airway remodelling is observed in both mild and severe asthma[3]. It is generally believed that an imbalance in T helper 1 (Th1) and T helper 2 (Th2) cells caused by an excessive increase in Th2 cells is the initiating factor of asthma[4].

There are three main mechanisms of airway remodelling: (1) EMT of the airway epithelium; (2) hypertrophy and proliferation of airway smooth muscle; and (3) subepithelial fibrosis caused by imbalance in degradation-deposition of extracellular matrix.

EMT is a process in which epithelial cells detach and transform into mesenchymal cells with migratory abilities. EMT of airway epithelial cells is one of the important factors leading to airway remodelling, and it also leads to subcutaneous fibrosis of the tracheal wall, which thickens the tracheal wall and eventually leads to airflow limitations[5]. EMT can be induced by a variety of cytokines, transcription factors, and inflammatory factors, such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), interleukin-6 (IL-6), and WNT. Among them, TGF-β is a key factor has been widely reported to upregulate the transcription factors Snail-1 and Snail-2 by modulating multiple signalling pathways, including mothers against decapentaplegic homologue (Smad), phosphatidylinositol 3 kinase (PI3K) and RAS, which ultimately lead to the EMT[6].

The Wnt/β-catenin signalling pathway is also critical in regulating the EMT in general, and its core is β-catenin. In cells, β-Catenin is complexed with anaphase promoting complex (APC)/axis inhibition protein (Axin)/glycogen synthase kinase 3 beta (GSK-3β) and cannot exert its activity, or it is phosphorylated and then degraded by ubiquitination. Under classical Wnt/β-catenin signalling, Wnt binds to the Frizzled/low-density lipoprotein receptor-related protein (LRP) receptor on the plasma membrane to activate the Wnt pathway, dissociating β-catenin from the complex and increasing its intracellular concentration. The increase in intracellular β-catenin leads to the upregulation of Snail-1 expression, which activates the EMT. β-catenin also activates the transcription of other downstream target genes to regulate cell proliferation and migration[7].

Abnormal hypertrophy and proliferation of airway smooth muscle cells play an important role in airway remodelling[8]. In addition, airway smooth muscle cells also secrete inflammatory factors and transforming factors. Of note, TGF-β induces increased secretion of connective tissue growth factor through the extracellular signal-regulated kinase (ERK) and Jun N terminal kinase (JNK) pathways. The hypertrophy and proliferation of smooth muscle cells are also regulated by the Wnt/β-catenin signalling pathway. When this pathway is activated, upregulated Snail-1 activates the expression of downstream genes, including the oncogene cellular myelocytomatosis (c-Myc), cyclin D1, and other target genes, resulting in hypertrophy and hyperplasia of smooth muscle[9].

Extracellular matrix (ECM) includes collagen, elastic fibres, reticular fibres, and mucopolysaccharides. The main components of ECM in lung tissue are type I and type III collagen. ECM plays an important role in the maintenance of normal cell function; however, when ECM is over-synthesized, it leads to small airway remodelling, which consequently causes airway obstruction and further exacerbates airway structural abnormalities and lung parenchymal damage[10]. The degradation and synthesis of ECM are regulated by matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of matrix metalloproteinase (TIMPs), respectively. MMPs are a Zn2+- and Ca2+-dependent protein superfamily that regulates ECM metabolism by decomposing ECM[11]. TIMPs form MMP-TIMP complexes, which prevent MMPs from being activated by zymogens, thus inhibiting their activity[12]. TIMPs

also inhibit the activity of activated MMPs and promote the proliferation of fibroblasts and collagen production[13].

Mesenchymal stem cells (MSCs) are pluripotent stem cells derived from the mesoderm and ectoderm at an early stage of development. MSCs can be differentiated into fat, bone, cartilage, muscle, tendon, ligament, nerve, liver, myocardial, endothelial, and other tissue cells under specific induction conditions *in vivo* or *in vitro*. In addition to repairing damaged tissues and replacing damaged functional cells, MSCs also have a very strong role in inhibiting inflammation and immunoregulation[14]. These findings have encouraged a series of attempts to treat graft-versus-host disease (GVHD) with MSCs. MSCs have low expression of major histocompatibility complex II (MHC-II) and T cell costimulatory factors, such as CD80, CD86, CD40, and CD40L. MSCs inhibit the immune cell response and T cell proliferation and activation by secreting cytokines such as IL-6, interleukin-10 (IL-10), and TGF-β1[15]. MSCs also increase regulatory T cells (Tregs) by secreting human leukocyte antigen G5 (HLA-G5)[16]. Therefore, MSCs escape rejection by the host immune system, making the application of heterogeneous allogeneic MSCs possible[17-19].

Part of the therapeutic effects of MSCs arises from the release of soluble paracrine factors. Exosomes are microvesicles that are secreted from cells and act as regulators of intercellular communication[20]. Increasing reports indicate that MSC-derived exosomes are one of the most attractive candidates for cell therapy applications in several diseases[21]. Exosomes range from 10-100 nm in size and contain proteins, mRNA, and miRNA molecules that can transfer between cells and affect protein expression in target cells[22]. MiRNAs in MSC-derived exosomes are important pathways that regulate the expression of target cell proteins. MiRNAs are endogenous noncoding RNAs that post transcriptionally regulate gene expression, and their role has been implicated in the pathogenesis of many diseases. Microvesicle-mediated miRNA transfer plays a role in a variety of lung diseases[23]. MiRNAs have been confirmed to correct the regulation of Wnt/β-Catenin signal transmission[24,25]. Therefore, we hypothesize that in combination with immunomodulatory and compensatory therapy, MSCs can reduce the EMT of epithelial cells and the proliferation and migration of smooth muscle cells by highly expressed miRNAs in exosomes that affect key proteins of the Wnt/β-catenin signalling pathway.

BML-284 is a modified pyrimidine. Recently, it has been reported that BML-284 activates the β-Catenin/transcription factor (TCF) gene in cultured cells. By examining its role in the developmental model of *Xenopus laevis*, BML-284 was identified as a small molecule agonist of the Wnt/β-catenin signalling pathway[26]. Therefore, we hypothesized that BML-284 could reverse the downregulation of the Wnt/β-catenin signalling pathway in airway epithelium caused by MSCs and MSC-derived exosomes. In this study, we used an asthma rat model to investigate the effect of MSCs and MSC-derived exosomes on Wnt/β-Catenin signal transduction and airway remodelling. Moreover, by combining the Wnt agonist BML-284 with MSC and MSC-derived exosome treatment, we further confirmed that MSCS and MSC-derived exosomes protect against the EMT and smooth muscle proliferation of airway epithelium by downregulating the Wnt signalling pathway.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats weighing 180-220 g were housed and maintained in the animal facilities of China Medical University. All animal protocols used were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. This investigation was approved by the Animal Ethics Committee of China Medical University Animal Centre. The rats were exposed to a 12 h/12 h light/dark cycle (50 lux in cages), the room temperature was 20±1°C, and the relative humidity was 40-60%. The rats were fed granular food and tap water. Then, 36 out of the 42 male rats were divided into 6 groups, and the remaining 6 rats were used to isolate bone marrow-derived MSCs. All rats were sacrificed by intraperitoneal injection of 200 mg/kg sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA).

Antigen Challenge

Ovalbumin (OVA) was used to sensitize the rats in the study. On the first day, the rats were sensitized with an intraperitoneal injection of 1 mL OVA (100 mg/mL; Solarbio, Beijing, China) + aluminium hydroxide emulsion (100 mg/mL; Ourchem, Shanghai, China), and on the eighth

day, the rats were sensitized with an intraperitoneal injection of 1 mL OVA emulsion (10 mg/mL; the control group was injected with 1 ml sterile saline). The sensitized rats were then placed in a 20 × 20 × 30 cm transparent closed container from the 15th-20th day were subjected to an atomizer. The rats were treated with atomized 5% OVA solution once per day for 30 min (the control group was treated with atomized sterile saline).

Isolation and Culture of Bone Marrow-Derived MSCs

The rats were sacrificed by intraperitoneal injection of 200 mg/kg sodium barbital. The femurs of the rats were isolated under aseptic conditions, scissors were used to cut the two ends of the thighbone, the marrow cavity was flushed with phosphate-buffered saline (PBS; BasalMedia, Shanghai, China) containing 2% fetal bovine serum (FBS; Sciencecell, Carlsbad, CA, USA), and the PBS containing the bone marrow cells was collected. The collected cells were cultured and maintained in T-25 flasks containing Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; BasalMedia, Shanghai, China) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Beyotime, Shanghai, China). On the third day, the culture media were changed to remove non-adherent cells. Then, we replaced the media every two days. When the cells reached 80-90% confluence, the adherent cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Beyotime, Shanghai, China) and passaged for 2-3 weeks. Subsequent passages and cell seeding were performed at a density of 2×10^5 cells/flask. Passage 3 (P3) MSCs were used for characterization analysis. The MSC phenotype was verified by surface marker expression of CD34, CD45, CD90, and CD105 using phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometric analyses were performed using a Miltenyi Biotec flow cytometer (MACSQuant® Analyser 10, Miltenyi Biotechnology, Bergisch Gladbach, Germany). FlowJo software (version 10.0) was used for data analysis.

Detection of Differentiation Ability of Bone Marrow-Derived MSCs

P3 rat bone marrow-derived MSCs were seeded in 9 wells of 6-well plates. Three wells of MSCs were cultured for 21 days with adipogenic differentiation medium composed of Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG; BasalMedia, Shanghai, China), 10% FBS, 1 μM dexamethasone (Solarbio, Beijing, China), 0.5 mM IBMX (Solarbio, Beijing, China) and 0.2 mM indomethacin (Med Chem Express, Monmouth Junction, NJ, USA). The medium was then replaced every two days. After induction culture, the cells were fixed with 4% paraformaldehyde (PFA; Solarbio, Beijing, China) and stained with oil red O (Ourchem, Guangzhou, China) staining solution for 30 min. After staining, the number of lipid droplets in the cells was observed under a microscope.

Three wells of MSCs were cultured for 21 days in osteogenic differentiation medium composed of Dulbecco's Modified Eagle's Medium-high glucose (DMEM-HG; BasalMedia, Shanghai, China), 10% FBS, 100 nM dexamethasone, 50 μM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM β-glycerophosphate disodium (Sigma-Aldrich, St. Louis, MO, USA). The medium was then replaced every two days. After induction culture, the cells were fixed with 4% PFA and stained with Alizarin Red S (Solarbio, Beijing, China) solution for 30 min. The number of calcium nodules was observed under a microscope.

Three wells of MSCs were cultured for 18 days with chondrogenic differentiation medium composed of DMEM-HG (BasalMedia, Shanghai, China), 10% FBS, 50 μg/ml ascorbic acid, 100 nM dexamethasone, 100 μg/mL ammonium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 40 μg/mL L-proline (Sigma-Aldrich, St. Louis, MO, USA), 50 mg/mL ITS Premix (BD Biosciences, San Jose, CA, USA) and 10 ng/mL TGF-β1 (ExCell Bio, Shanghai, China). The medium was then replaced every two days. After induction culture, the cells were fixed with 4% PFA. The cells were then incubated with the appropriate diluted anti-type II collagen primary antibody (ProteinTech, Rosemont, IL, USA) for 2 h at 37°C, and then HRP-conjugated IgG secondary antibody (ProteinTech, Rosemont, IL, USA) was added. The cells were incubated at 37°C for 1 h, followed by incubation with diaminobenzidine (DAB) solution (Solarbio, Beijing, China). The collagen type II immunoreactivity level of the cells was observed under a microscope.

Isolation of MSC-Derived Exosomes

P3 rat bone marrow-derived MSCs were cultured in serum-free DMEM/F12 medium for 48 h at 37°C. The cell culture supernatant was cen-

trifuged at 3000 g for 15 min, the supernatant was retained, and the precipitate was removed. The remaining supernatant was transferred into a 100 KD Amicon® Ultra-15 Centrifugal Filter Unit (Millipore, Billerica, MA, USA) and centrifuged at 4000 g for 30 min. An Exoquick TC exosome extraction kit was used with the concentrated liquid to purify the extracted exosomes. The bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) was used to quantify the extracted exosomes. The morphological information (15000×) of exosomes was collected by transmission electron microscopy (TEM; Hitachi HT7800, Japan). Total proteins were extracted from the MSC-derived exosomes by radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), and the proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). For exosome analysis, the membranes were incubated with anti-CD9, anti-CD63 (Abcam, Cambridge, MA, USA), and anti-CD81 (Abcam, Cambridge, MA, USA) primary antibodies at 4°C for 12 h. Horseradish peroxidase (HRP)-conjugated secondary antibody (ProteinTech, Rosemont, IL, USA) was then used to detect the bands. β-actin antibody (ProteinTech, Rosemont, IL, USA) was used as a loading control.

MSC and MSC-Derived Exosome Treatment and Wnt Agonist Intervention

The rats were divided into 6 groups as follows: (A) vehicle control group, (B) model group, (C) MSC injection group, (D) MSC-derived exosome injection group, (E) MSC+BML-284 injection group, (F) MSC-derived exosome+BML-284 injection group.

Injection method: on the 15th day of modelling, before allergen challenge, 5×10^6 MSCs in 1 mL PBS were injected into the tail vein of each experimental rat in groups C and E, and 50 μg exosomes in 1 mL PBS was injected into the tail vein of each experimental rat in groups D and F. Each rat in groups E and F was additionally injected with 500 μL BML-284 (2 mg/mL, diluted with 20% dimethyl sulfoxide + 80% PBS) (Med Chem Express, Monmouth Junction, NJ, USA) 30 min after intravenous injection of MSCs or 50 μg exosomes[27]. Each rat in groups A and B was injected with sterile PBS in the tail vein. After the injection, each group of rats continued to be challenged with aerosolized target antigens.

Bronchoalveolar Lavage Fluid (BALF) Isolation

After the animal experiment, the rats were sacrificed by intraperitoneal injection of 200 mg/kg sodium barbital. The lungs of each rat were lavaged 3 times with 2 mL of PBS, and then the BALF was collected. The BALF cell composition of each rat was determined by using a blood cell analyser.

Chemical Staining Analysis of Lung Tissue Sections

After lavage, lung tissue was isolated and fixed with 10% neutral formalin (Ourchem, Shanghai, China) for 48 h, dehydrated, and embedded in paraffin (SCR, Shanghai, China). Lung sections at a thickness of 4 μm were stained with haematoxylin and eosin (H&E) to assess the level of lung inflammation. Masson staining (Solarbio, Beijing, China) was used to evaluate peribronchial collagen deposition.

Western Blotting (WB)

Lung homogenates with bronchi were prepared for immunoblotting. Proteins were separated by SDS-PAGE. After transfer, the PVDF membranes were incubated with primary antibodies against E-cadherin (ProteinTech, Rosemont, IL, USA), vimentin (ProteinTech, Rosemont, IL, USA), WNT (ProteinTech, Rosemont, IL, USA), Frizzled (ProteinTech, Rosemont, IL, USA), GSK-3β (ProteinTech, Rosemont, IL, USA), p-Ser-GSK-3β (Bioss, Beijing, China), p-Tyr-GSK-3β (Bioss, Beijing, China), β-catenin (ProteinTech, Rosemont, IL, USA), Snail1 (ProteinTech, Rosemont, IL, USA), and c-Myc (ProteinTech, Rosemont, IL, USA). Then, HRP-conjugated secondary antibodies (ProteinTech, Rosemont, IL, USA) were used to detect the bands. The anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (ProteinTech, Rosemont, IL, USA) was used as a loading control for protein normalization.

Real Time-Polymerase Chain Reaction (PCR) Analysis of mRNA

Lung tissue containing bronchi was homogenized, and the total RNA of the tissue was extracted by using a total RNA extraction kit (Solarbio, Beijing, China). mRNA was reverse transcribed into cDNA as soon as possible using a reverse transcription kit (Solarbio, Beijing, Chi-

na). The sequences of WNT, β-catenin, E-cadherin, Vimentin, GSK-3β, Snail1, c-Myc, and cyclin D1 mRNAs were found in the National Centre for Biotechnology Information (NCBI) database. Primers were designed to detect the relative expression of these mRNAs. We used GAPDH as a control for normalization. Real-time PCR was performed in a 20 µL reaction containing SYBR Green and a pair of primers for one of the above mRNAs or controls on a real-time PCR system (Bio-Rad, Hercules, CA, USA). After 40 cycles of 2-step amplification, we obtained the threshold cycle (Ct) value of each 20 µl reaction. The expression levels were quantified by applying the comparative Ct method and calculating $2^{-\Delta\Delta Ct}$. Relative expression levels of the target genes were normalized to the expression of GAPDH in each individual sample. Gene-specific primers are as follows: GAPDH sense: 5'-GCTGAGA ATGGGAAGCTGGT-3', anti-sense: 5'-GCCTTCTCCATGGTGGTGAA-3'; E-cadherin sense: 5'-ATCCTGGCCCTCCTGATTCT-3', anti-sense: 5'-CGGGTATCGTCATCTGGTGG-3'; Vimentin sense: 5'-CTCTGGTTGACACCCACTCC-3', anti-sense: 5'-AAGGTCATCGTGGTGCTGAG-3'; WNT sense: 5'-CGTTGCTGTCCCTGTGGTAT-3', anti-sense: 5'-CAGGTGTG GTGGTTAGGGAC-3'; β-Catenin sense: 5'-TCGGTTGAGCTGACCAGTTC-3', anti-sense: 5'-GTCCTGGCGATATCCAAGGG-3'; Frizzled sense: 5'-CCGCACCATCATGAAGCATG-3', anti-sense: 5'-AGTAGCAGGCGATGACGATG-3'; GSK-3β sense: 5'-GGCTAACA CCAC TGGAAGCT-3', anti-sense: 5'-GAAGAGGGCAGGTGTGTCTC-3'; Snail-1 sense: 5'-AAGATGC ACATCCGAAGCCA-3', anti-sense: 5'-CAGTGGGAGCAGGAGAAAGG-3'; and c-Myc sense: 5'-CAACAACCGCAAATGCTCCA-3', anti-sense: 5'-AGCTACGCTTCAGCTCGTTT-3'.

Statistical Analysis

The results are expressed as the mean ± standard deviation. GraphPad Prism 5 (LaJolla, CA, USA) was used for data representation. Differences between two groups were analysed by using Student's *t*-test. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) followed by a post-hoc test (least significant difference). A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

Bone Marrow Derived MSC Characterization

Flow cytometry analysis showed that the P3 bone marrow-derived MSCs were CD90- and CD105-positive and CD34- and CD45-negative (Figure 1A). Bone marrow-derived MSCs were cultured in three differentiation media for 18-21 days. Positive staining of collagen type II

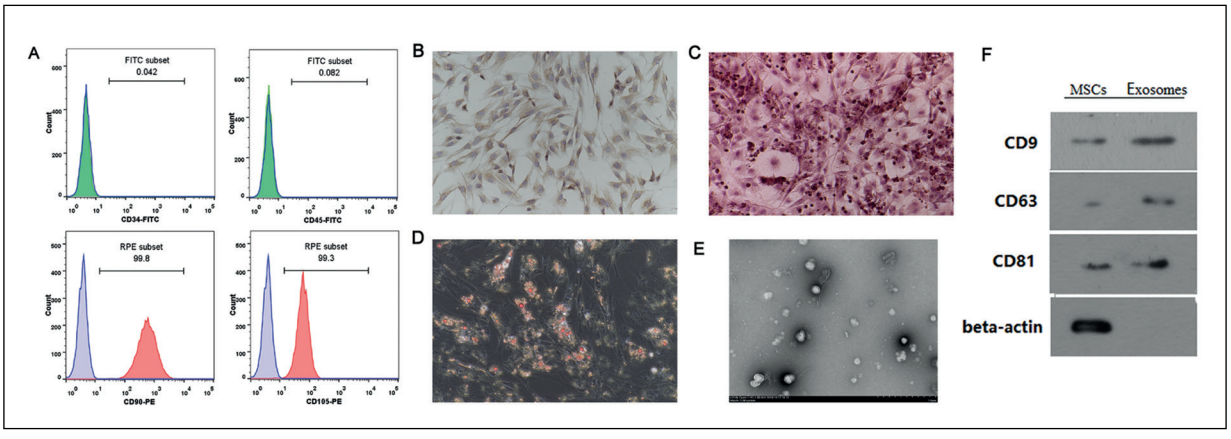

Figure 1. Characterization and functional validation of rat bone marrow-derived mesenchymal stem cells and their exosomes. **A,** Phenotypic analysis of cell surface antigens of rat bone marrow-derived mesenchymal stem cells (BMSCs). Fluorescent histograms of isotype controls are shown in blue. **B,** Immunohistochemical staining of type II collagen in BMSCs after chondrogenic induction for 18 days (magnification: 200×). **C,** Alizarin red S staining of BMSCs after osteogenic induction for 21 days (magnification: 200×). **D,** Oil red O staining of BMSCs after adipogenic induction for 21 days (magnification: 200×). **E,** Cup-shaped morphology of purified Exos was assessed by transmission electron microscopy. Scale bar = 1.0 µm. **F,** Immunophenotype of bone marrow MSCs and MSC-derived exosomes. Cells and exosomes were labelled with antibodies specific for the indicated rat surface antigens and then assessed by Western blot analysis. β-actin was used as a loading control for cells.

demonstrated that MSCs differentiated into chondrocytes (Figure 1B). Alizarin Red S staining showed positive calcification nodules and demonstrated successful differentiation of MSCs into osteoblasts (Figure 1C). The oil droplets strained by oil red O in cells indicated that MSCs differentiated into adipocytes (Figure 1D).

Quality Verification of MSC-Derived Exosomes

After exosome extraction, TEM analysis showed that the exosome diameters were in the normal range (Figure 1E). The levels of the exosome markers CD9, CD63, and CD81 were higher in exosomes than in the total protein extract of bone marrow-derived MSCs, and the expression of β-actin was negative (Figure 1F), as shown by Western blotting.

MSC and MSC-Derived Exosome Treatment and Wnt Agonist Intervention in Infiltrating Cells in the Lungs of Model Rats

The total cells, lymphocytes, eosinophils, and neutrophils in BALF in the model group were significantly increased compared to those of the vehicle control ($p<0.001$), while these parameters in asthma model rats that were treated with MSCs and MSC-derived exosomes during sensitization were significantly reduced ($p\leq0.001$). Subsequent addition of the Wnt agonist BML-284 to the latter groups recovered the number of total cells, lymphocytes, eosinophils, and neutrophils ($p\leq0.001$; Figure 2).

Lung Histology of Model Rats After the Treatment with MSCs and MSC-Derived Exosomes and Intervention with the Wnt Agonist

Model rats with allergic airway inflammation were successfully established by repeated OVA challenge. H&E staining showed evident inflammatory cell infiltration and goblet cell hyperplasia in the peribronchial tissue of the model rats. Treatment with MSCs and MSC-derived exosomes significantly decreased airway inflammation and the proliferation of goblet cells in the lung, while the subsequent addition of the Wnt agonist BML-284 caused the number of airway inflammatory cells to increase significantly compared with those of the MSC and MSC-derived exosome treatment groups (Figure 3). Masson staining showed that collagen deposition in the model group was much higher than that in the control group, while MSCs and MSC-derived exosomes significantly reduced collagen deposition in the lungs of model rats. Similar to what was observed in the infiltrating cells, the addition of the Wnt agonist BML-284 reversed the effects of MSCs and MSC-derived exosomes on collagen deposition (Figure 3).

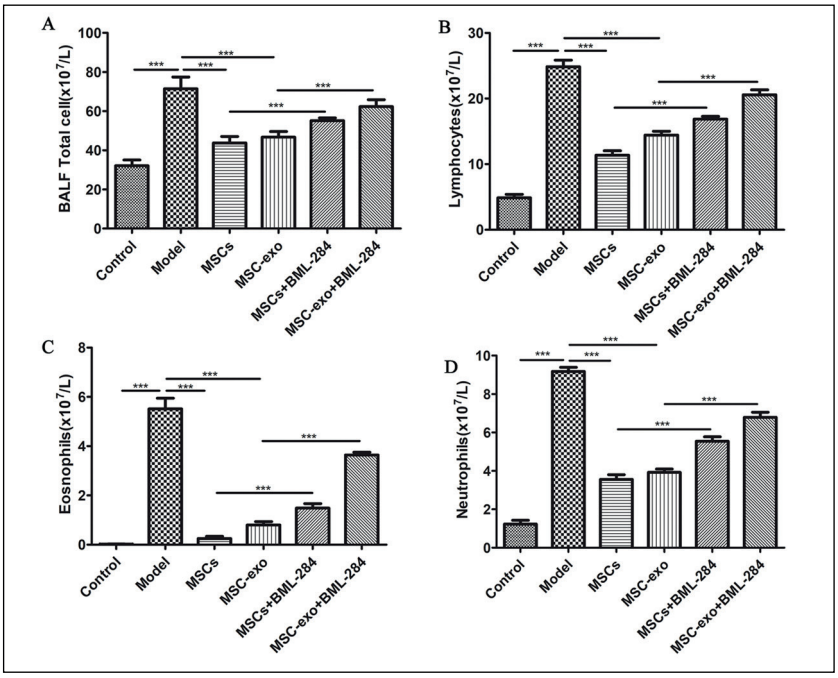

Figure 2. The number of total cells (**A**), lymphocytes (**B**), eosinophils (**C**) and neutrophils (**D**) in bronchoalveolar lavage fluid (BALF) in the different groups. The results are presented as the mean ± SEM (n = 5). Statistical analysis was performed with one-way ANOVA followed by Tukey's test. ** $p < 0.01$, *** $p < 0.001$.

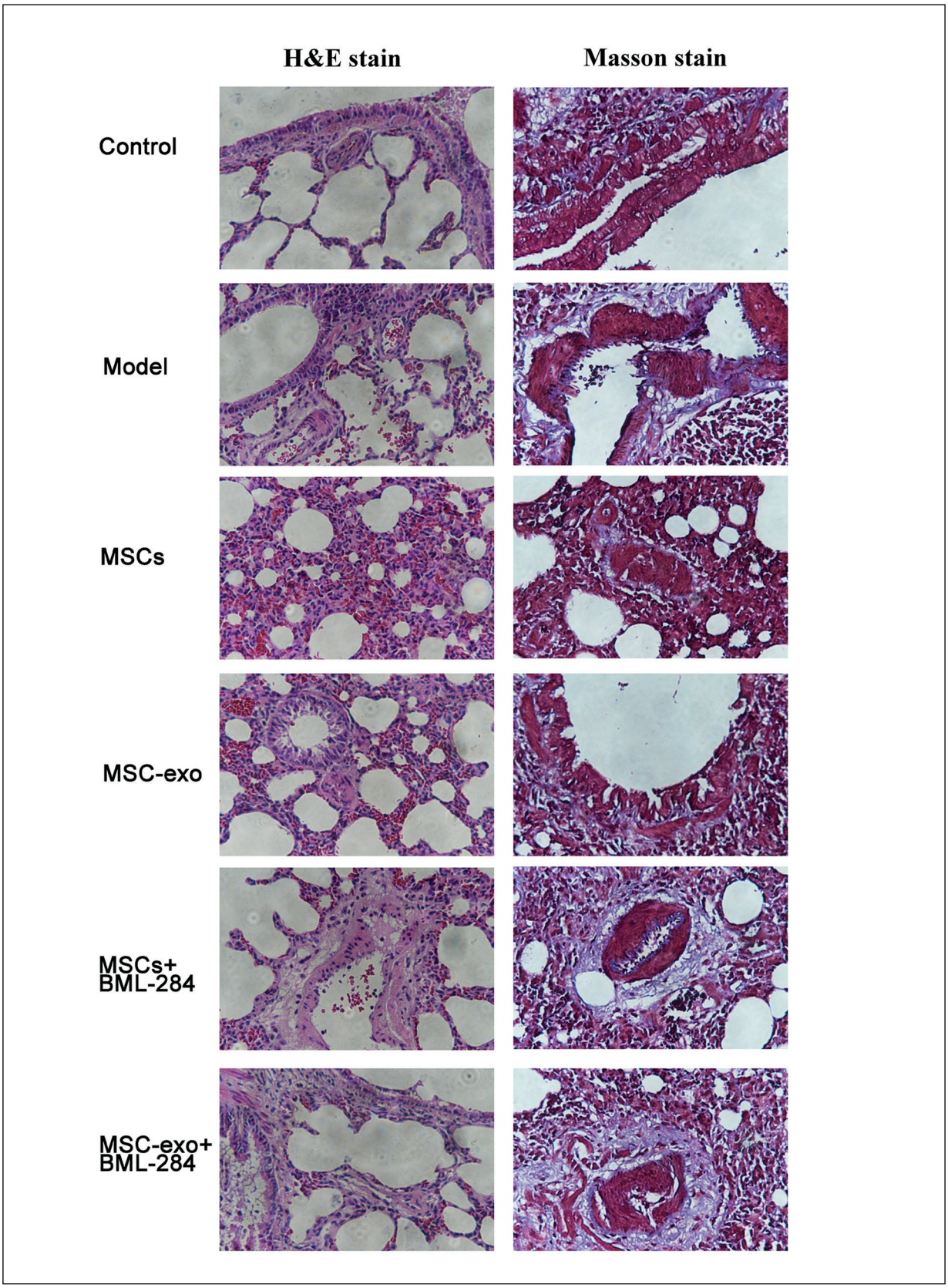

Figure 3. Histopathological assessment using haematoxylin and eosin (H&E) and Mason's staining (magnification: 200×).

Effects of MSCs, MSC-Derived Exosomes and the Wnt Agonist on Wnt/β-Catenin Pathway Proteins and Related Protein Levels in Lung Tissue

To further demonstrate the mechanism of MSC and MSC-derived exosome treatment on chronic allergic airway inflammation, we evaluated several EMT-related proteins (E-cadherin and Vimentin), Wnt/β-catenin pathway proteins (WNT, β-Catenin, Frizzled, GSK-3β, p-Ser-GSK-3β, and p-Tyr-GSK-3β), and Wnt/β-catenin pathway downstream proteins (Snail1 and c-Myc). The vimentin level was significantly increased in the model group compared with that of the control group ($p \leq 0.001$). MSCs and MSC-derived exosomes significantly reduced vimentin levels ($p \leq 0.001$), while the subsequent addition of the Wnt agonist BML-284 caused the level of vimentin to increase again ($p \leq 0.05$). The trend in E-cadherin was opposite to that of vimentin, which suggests that OVA-induced asthma promotes the EMT in airway epithelial cells, MSCs and MSC-derived exosomes inhibit this process, and the addition of Wnt agonists activate this suppressed EMT (Figure 4A-4C).

WB showed that WNT, Frizzled, p-Ser-GSK-3β, and β-catenin in the lungs of the asthma model were significantly upregulated compared with those of the control group and were effectively suppressed by the treatment with MSCs and MSC-derived exosomes ($p \leq 0.001$). This effect was offset by the addition of BML-284 ($p \leq 0.05$; Figure 4D, 4E, 4G, and 4I). Similar patterns of protein regulation by asthma, MSC, and MSC-derived exosome treatment, and BML-284 were also observed in Wnt/β-Catenin pathway downstream proteins (Snail-1 and c-Myc; Figure 4J and 4K). The protein expression of p-Tyr-GSK-3β was different from that of the other proteins because it is a negative regulator in

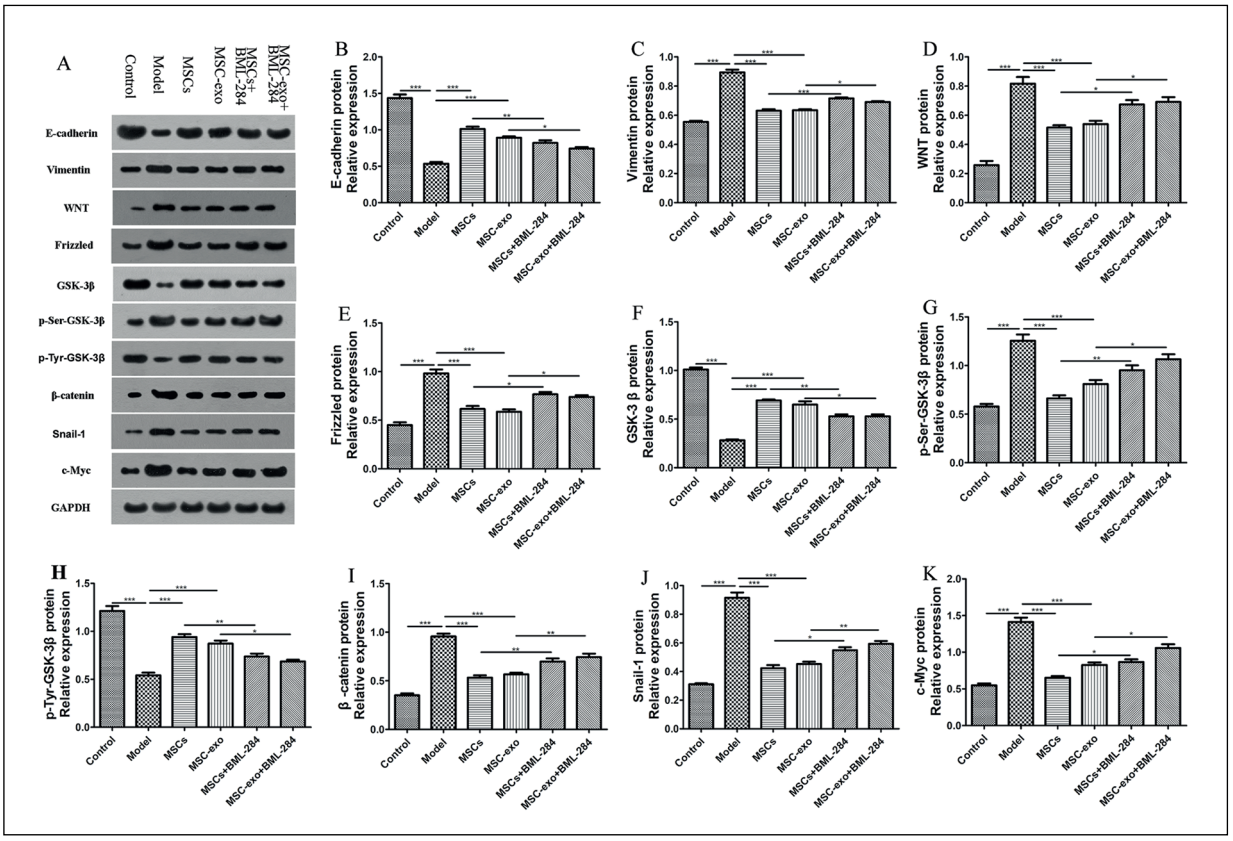

Figure 4. The expression levels of EMT-related proteins, Wnt/β-catenin pathway proteins and Wnt/β-catenin pathway downstream proteins in lung tissue. **A,** Western blot analysis was used to detect the expression of E-cadherin, Vimentin, WNT, β-Catenin, Frizzled, GSK-3β, p-Ser-GSK-3β, p-Tyr-GSK-3β, Snail-1 and c-Myc. **B-K,** The relative expression of E-cadherin, Vimentin, WNT, β-Catenin, Frizzled, GSK-3β, p-Ser-GSK-3β, p-Tyr-GSK-3β, Snail-1 and c-Myc in the different groups. The relative densities of all bands were analysed and normalized to the GAPDH level. The results are presented as the mean ± SEM (n = 3). Statistical analysis was performed with one-way ANOVA followed by Tukey's test. *$p < 0.05$** $p < 0.01$, *** $p < 0.001$.

the Wnt/β-catenin pathway (Figure 4I). GSK-3 β usually binds to β-Catenin in its phosphorylated state (p-Tyr-GSK-3β) to inhibit activation of the Wnt pathway (Figure 4F and 4H).

Effects of MSCs, MSC-Derived Exosomes and the Wnt Agonist on Wnt Pathway mRNA and Related mRNA Levels in Lung Tissue

The results of real-time PCR were consistent with those of WB. The mRNA expression levels of Vimentin, WNT, Frizzled, β-catenin, Snail1, and c-Myc were significantly increased in the asthma model group. The mRNA expression of E-cadherin was opposite to that of the protein (Figure 5A). MSCs and MSC-derived exosomes reduced the expression of these genes, while the addition of the Wnt agonist increased the expression of these genes, which had been reduced (Figure 5B, 5C, 5D, 5F, 5G, and 5H). The mRNA expression of GSK-3β was opposite to that of the protein (Figure 5E).

Discussion

Allergic asthma is a chronic inflammatory disease. An imbalance in Th1 and Th2 cells is considered to play an important role in the development of asthma[28]. This disorder may result from deficiencies in Tregs that control lung immune responses[29]. Palomares et al[30] have indicated that Tregs play an important role in maintaining tolerance in the immune system. Glucocorticoids are currently the first-line treatment for asthma and effectively improve the condition of patients, but their clinical effects on patients with severe airway remodelling are not ideal[31]. Moreover, the long-term use of glucocorticoids may have adverse effects on the further treatment of asthma[32]. Therefore, there is an urgent need for a strategy to improve the long-term treatment effect while minimizing the side effects.

In this study, we found that injection of MSCs and MSC-derived exosomes improved the pathophysiological characteristics and attenuated chronic airway inflammation. Our results indicated that MSCs and MSC-derived exosomes significantly reduced the total cells, lymphocytes, eosinophils, and neutrophils in OVA-sensitized and challenged rats. Treatment with MSCs and MSC-derived exosomes also inhibited airway remodelling in rats that were exposed to repeated allergens. However, injection of the Wnt pathway agonist reduced the therapeutic effects of MSCs and MSC-derived exosomes.

Knight et al[33] have shown that MSCs can be differentiated into airway epithelial cells to re-

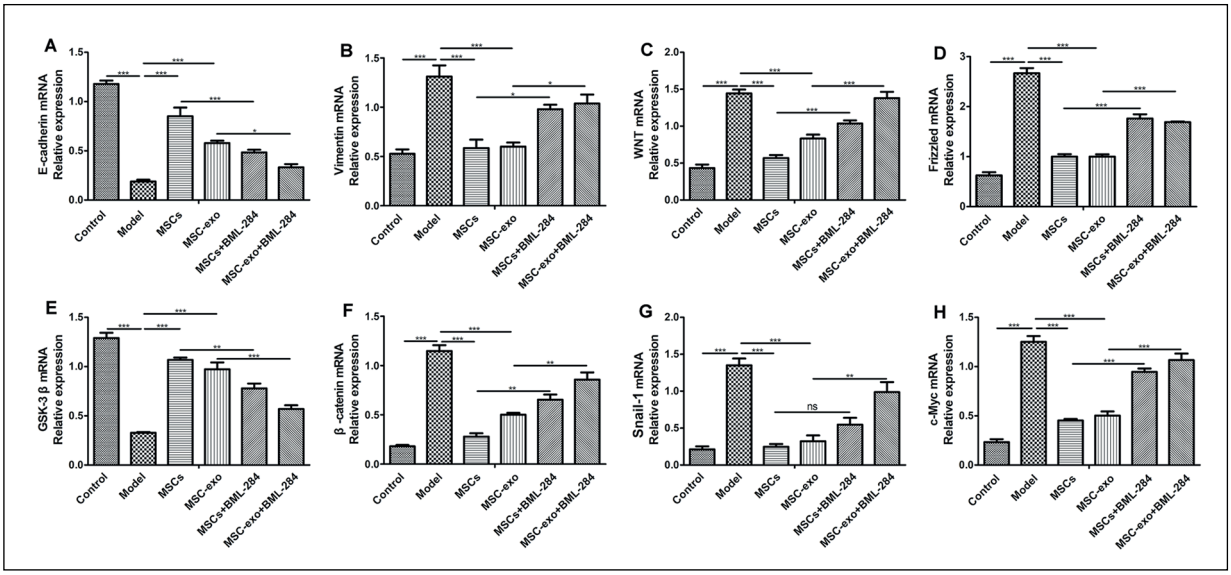

Figure 5. Gene expression levels of EMT-related proteins, Wnt/β-catenin pathway proteins and Wnt/β-catenin pathway downstream proteins in lung tissue. Real-time PCR analysis was used to quantify the mRNA levels of E-cadherin (**A**), Vimentin (**B**), WNT (**C**), β-Catenin (**D**), Frizzled (**E**), GSK-3β (**F**), Snail-1 (**G**) and c-Myc (**H**) in the different groups. Relative expression levels of the target genes were normalized to the expression of GAPDH in each individual sample. The results are presented as the mean ± SEM (n = 3). Statistical analysis was performed with one-way ANOVA followed by Tukey's test. *$p < 0.05$** $p < 0.01$, *** $p < 0.001$.

pair airway damage caused by asthma. MSCs also inhibit the activation and quantity of various immune cells through their powerful immunoregulatory effects[34]. MSCs also reduce the levels of various Th2 cytokines, such as interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13) and IgG and IgE immunoglobulins, thus inhibiting the inflammatory response caused by asthma[35]. MSCs significantly inhibit airway remodelling induced by asthma and directly inhibit the development of tracheal EMT and the proliferation of smooth muscle cells. The exosomes secreted by MSCs directly inhibit the expression of specific proteins in related pathways. In this study, we demonstrated that rat MSCs regulate airway remodelling by MSC-derived exosomes, which modulate the expression of signalling molecules of the Wnt/β-catenin signalling pathway.

The histological features of chronic asthma are goblet cell hyperplasia, mucinous gland hypertrophy, subepithelial fibrosis, and smooth muscle cell hyperplasia and hypertrophy[36,37]. We observed that MSCs and their exosomes reduced collagen deposition and airway thickening, which proved the therapeutic effect of MSCs and their exosomes on chronic allergic airway inflammation. After OVA sensitization, injection of MSCs and their exosomes significantly reduced the expression of Snail-1, and mRNA and protein expression of all positive regulatory indicators of the Wnt/β-Catenin pathway were also decreased. The Wnt/β-Catenin pathway is one of the important pathways related to the EMT and airway remodelling. After activation, snail-1 upregulates the expression of downstream genes such as c-Myc. These genes regulate the proliferation and migration of smooth muscle cells. In our research, after the injection of MSCs and MSC-derived exosomes, the subsequent addition of a small molecule activator of Wnt reduced the therapeutic effect, leading to reactivation of the Wnt pathway and exacerbation of airway remodelling. Therefore, it has been proven that the mechanism by which MSCs can treat asthma is by exosome-mediated modulation of the Wnt/β-Catenin pathway to affect the EMT of airway epithelial cells and the proliferation of airway smooth muscle cells.

MSCs are increasingly found to have anti-inflammatory effects in a wide range of inflammatory and immune-mediated disease models[38,39]. MSCs provide very good clinical effects without a large amount of homing to the target organ and without differentiation, although the injected cells will disappear after a short time. If MSCs survive for a long time, the detailed mechanism of the long-term effects of MSCs on chronic allergic airway inflammation should be further studied in the future. Galipeau et al[40] have shown that MSC-mediated protective effects may be related to a decrease in macrophage immune-related abilities after apoptotic MSC fragments are engulfed by macrophages. We provide a potential therapeutic approach to reverse airway remodelling to address airway airflow restriction caused by asthma. Further studies are needed to confirm the therapeutic effect and mechanism of exosomes secreted by MSCs. In addition, MSCs may have multiple mechanisms to inhibit airway remodelling in chronic allergic airway inflammation, which have yet to be elucidated.

Conclusions

We demonstrated that MSCs and their exosomes inhibit the Wnt/β-Catenin pathway in the lung to effectively inhibit chronic allergic airway inflammation. The addition of a Wnt activator reduced the therapeutic effect of MSCs, which confirmed this hypothesis. Cells and exosomes may be potential therapeutic candidates for the treatment of long-term asthma airway remodelling. However, further research is needed to fully reveal the mechanism of the immunomodulatory effect of MSC-derived exosomes to support the clinical application of MSCs and MSC-derived exosomes in chronic allergic airway inflammation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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