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Stepwise differentiation of airway epithelial cells from human tonsil-derived mesenchymal stem cells

Ha Yeong Kim[1], Jiin Yang[1], Han Su Kim[1] and Soo Yeon Jung[1*]

Abstract

Background Airway defects, often resulting from tumor resection, trauma, or infection, pose significant treatment challenges owing to the intricate, multilayered structure of the airway. Successful recovery depends on reconstructing the respiratory epithelium, the lining next to the cartilage. Although autologous epithelial or progenitor cells are used to reconstruct the epithelium, they are not ideal for regeneration because of difficulties in expansion and differentiation in vitro. In this study, we developed an alternative approach to respiratory epithelial regeneration using human tonsil-derived mesenchymal stem cells (TMSCs) to induce epithelial cell differentiation through a stepwise process.

Methods TMSCs were isolated from the human tonsillar tissues of patients undergoing tonsillectomy and differentiated into airway epithelial cells following human embryonic development. To generate airway epithelial cells, TMSCs were exposed to various chemical agents or protein combinations during a 4-step process.

Results We observed that TMSCs can be induced into the definitive endoderm (DE) with a low concentration of activin A, activating the Nodal/TGF-β signaling pathway. Subsequently, a combination of growth factors regulating BMP, TGF-β, and Wnt signaling induces the differentiation of TMSC-derived DE cells into anterior foregut endoderm, identified by upregulating PAX9, SOX2, and GATA3 gene expression. In the final 3–4 steps, an environment rich in Wnt and FGFs differentiated TMSCs into airway epithelial cells through lung progenitor cells, as evidenced by the increased gene expression of lung progenitor cell markers (NKX2-1), airway cell markers (KRT5), and ciliated cell markers (FoxJ1). Specifically, TMSC-derived airway epithelial cells exhibited a columnar epithelial structure resembling an F-actin filament structure.

Conclusions Our results demonstrated that TMSC-derived airway epithelial cells can be generated through stepwise differentiation and represent a potential alternative for the functional recovery of respiratory defects.

Keywords Respiratory dysfunction, Epithelial cells, Tonsil-derived mesenchymal stem cells, Stepwise differentiation

Introduction

The airway comprises both the upper (nasal cavity, pharynx, and larynx) and lower (trachea, bronchi, bronchioles, and alveoli) respiratory tracts, which are lined by specialized epithelial cells forming the respiratory epithelium [1]. This epithelial layer serves as a critical barrier, protecting the host from inhaled pathogens and environmental insults [2]. Because of constant exposure to airborne particles, microbes, and toxic agents, the airway epithelium is highly vulnerable to injury. Such damage

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can occur in a range of diseases and conditions, including infections, chronic inflammation, cancer, or trauma [3, 4]. Although the airway epithelium possesses some regenerative capacity through resident basal or stem-like cells, its ability to fully repair is limited in cases of extensive or repetitive injury, often resulting in long-term tissue dysfunction.

To address such defects, autologous airway epithelial cells have been explored for regenerative therapy. These cells can be obtained via bronchoscopic brushing, a minimally invasive procedure that allows for the collection of immunologically compatible cells [5]. However, the clinical application of these cells is limited by low cell yield and restricted proliferative capacity [6]. To overcome these limitations, stem cells have gained increasing attention because of their high expandability and differentiation potential into tissue-specific lineages.

Human induced pluripotent stem cells (iPSCs) are a powerful tool for regenerative medicine because they differentiate into all three germ layers [7]. When guided by developmental signaling cues, iPSCs can be directed toward airway progenitor fates [8]. Nonetheless, iPSC-derived epithelial cells often exhibit immature, fetal-like features [9, 10]. For example, several studies have shown that most iPSC-derived airway epithelial cells resemble cells from the second trimester of fetal development and lack mature markers such as MHC class II molecules [11].

Mesenchymal stem cells (MSCs) have recently emerged as another promising cell source for airway regeneration. Compared to iPSCs, MSCs offer several advantages, including low immunogenicity, lack of tumorigenicity, and ease of isolation from various tissues [12]. Additionally, they possess a high capacity for self-renew, thereby facilitating their maintenance in long-term culture [13, 14]. Although their differentiation potential is more limited than that of iPSCs, MSCs can still be induced to acquire tissue-specific characteristics under optimized conditions. Among various MSC sources, tonsil-derived MSCs (TMSCs) are particularly attractive because of their non-invasive accessibility and high yield from discarded tonsil tissues after tonsillectomy [15]. TMSCs exhibit robust proliferative capacity, producing up to 10^8–10^9 cells, and have been shown to differentiate into mesodermal, ectodermal (e.g., neuron-like cells), and endodermal (e.g., parathyroid hormone- and insulin-releasing cells) lineages [16–18].

Although previous studies have attempted to induce epithelial differentiation from MSCs using co-culture with airway epithelial cells, the efficiency has remained low, and mature epithelial features have not been well characterized [19–21]. Recent advances indicate that stepwise differentiation protocols mimic stage-specific embryonic signaling cues and may enhance lineage-specific outcomes in MSCs. For example, Li et al. successfully generated pancreatic progenitor cells from human adipose-derived MSCs (A-MSCs) using a stepwise protocol involving sequential activation of signaling pathways with factors such as activin A, Wnt3a, retinoic acid (RA), Dickkopf-1 (DKK1), and FGF2 [22]. Based on these findings, we hypothesized that a similar stepwise strategy could guide TMSCs toward differentiation into airway epithelial cells. Therefore, this study aimed to establish a stepwise differentiation protocol for airway epithelial induction from TMSCs and to characterize the cellular and molecular profiles of each intermediate stage.

Materials and methods

Cell expansion

TMSCs were isolated from the tonsil tissues of patients younger than 10 years who underwent tonsillectomy with informed written consent as previously described [15]. The protocol was approved by the Institutional Review Board (EUMC-2019-08-030) of Ewha Womans University Mokdong Hospital, Korea. The isolated TMSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing low glucose (1000 mg/L) (DMEM-LG; Welgene Inc., Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Young In Frontier, Seoul, Korea), 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 μg/mL amphotericin B at 37 °C in a humidified CO_2 incubator. TMSCs were used between passages 4 – 7 for all experiments.

PCR analysis

For reverse transcription-PCR (RT-PCR) analysis, mRNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was synthesized using 200 U of SuperScript™ III reverse transcriptase, 2 mM dNTP, and 10 pmol of oligo-dT primer, following the manufacturer's protocol (Thermo Fisher Scientific). RT-PCR was performed using a PCR thermal cycler under the reaction conditions specified for each primer (Supplementary Material 1).

In quantitative real-time polymerase chain reaction (qRT-PCR), conducted as a separate experiment, the synthesized cDNA from cells was used to determine the gene expression using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) and CFX96 Real-Time System (Bio-Rad). All primer sequences and reaction conditions are listed in Supplementary Material 1.

Flow cytometry

For immunophenotype analysis, cells were collected and stained for 30 min at 4 °C with the following antibodies (BD Biosciences, San Jose, CA,

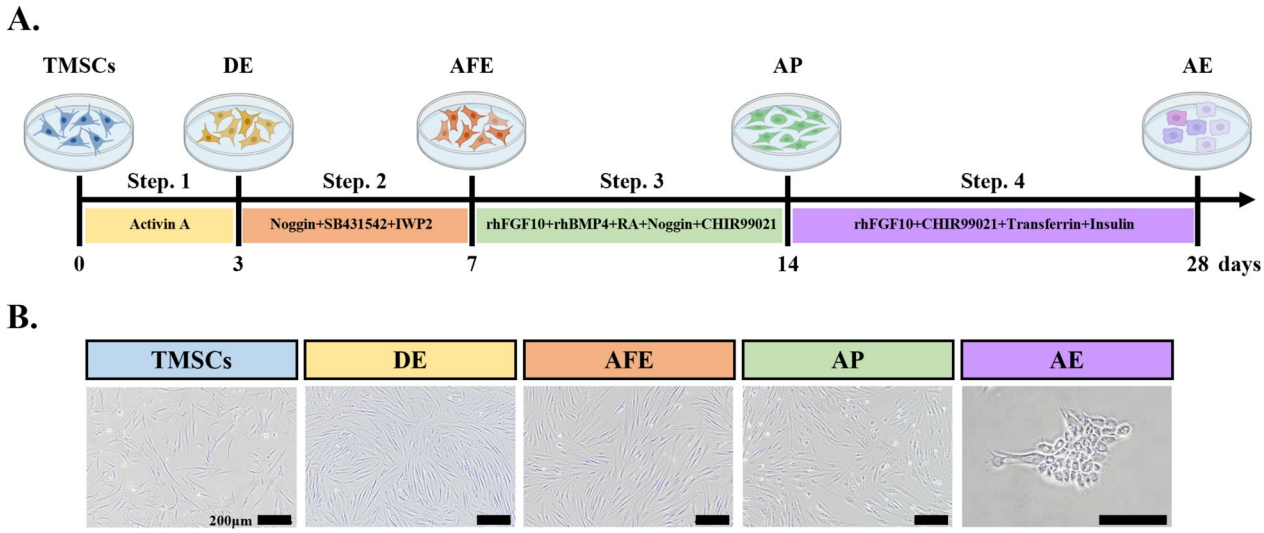


Fig. 1 The stepwise differentiation into airway epithelial cells using TMSCs. **A** Schematic diagram of the procedure to generate airway epithelial cells from TMSCs. **B** Microscopic images are taken of cells at each stage of the differentiation process. Scale bar = 200 μm

USA)—phycoerythrin-conjugated anti-human antibodies against CD14, CD34, and CD73 or fluorescein isothiocyanate-conjugated anti-human antibodies against CD45, CD90, and CD105. The stained cells were washed and examined using an ACEA NovoCyte flow cytometer (Agilent Technologies Inc., Santa Clara, CA, USA).

Tri-lineage differentiation of TMSCs

TMSCs were used to assess the level of tri-lineage differentiation, including adipogenesis, chondrogenesis, and osteogenesis. Cells were seeded in 24-well plates at 3×10^4 cells/well and differentiated using a commercially available adipogenic, chondrogenic, or osteogenic differentiation medium (Thermo Fisher Scientific) for 2 weeks. After differentiation, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed twice with Dulbecco's phosphate-buffered saline (DPBS). To confirm the differentiation capacity, cells were stained with Oil Red O (adipogenesis), Alcian blue (chondrogenesis), or Alizarin red S (osteogenesis) solution. The stained cells were visualized under a microscope in at least four randomly selected fields. The visualized images were converted into red, green, and blue stacks to quantify the level of differentiation using the ImageJ software.

Differentiation of TMSCs into airway epithelial cells

TMSCs were seeded in a 60-mm cell culture dish at a density of 3.5×10^5 cells were induced to differentiate into airway epithelial cells in four steps after 24 h (Fig. 1A). Step 1 involved the induction into DE cells. The cells were washed with DPBS (Welgene Inc.) and treated with 0, 5, 10, and 100 ng/mL activin A in DMEM-LG supplemented with 0 or 10% serum for 3 days.

In step 2, cells were induced into anterior foregut endoderm (AFE) by switching to media containing 200 ng/mL Noggin, 10 μM SB431542, and 1 μM IWP2 in DMEM-LG supplemented with 0, 2, 5, or 10% serum. Subsequently, the cells were incubated for 4 days at 37 °C in a humidified CO_2 incubator.

In step 3, the AFE-induced cells were differentiated into lung progenitor cells using two different media compositions. The DM1 group consisted of 10 ng/mL FGF10, 10 ng/mL BMP4, 0.5 μM RA, and 1 μM CHIR99021 in serum-free DMEM-LG. The DM2 group consisted of 20 ng/mL FGF10, 20 ng/mL BMP4, 0.5 μM RA, 1 μM CHIR99021, and 100 ng/mL Noggin in serum-free DMEM-LG. Step 3 was maintained for 7 days, with the medium replaced every 3–4 days.

To generate airway epithelial cells, the cells were replated onto a new cell culture dish in DMEM-LG supplemented with 0.2% serum, 10 ng/mL FGF10, 2 μM CHIR99021, 2.5 μg/mL insulin, and 2.5 μg/mL transferrin. The cells were incubated for 14 days, with the medium replaced every 3–4 days.

Western blot analysis

TMSCs were lysed using cold lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, and Protease Inhibitor Cocktail™ (Roche, Basel, Switzerland). The protein concentration of the lysates was measured using a bicinchoninic acid

protein assay, and protein loading samples were prepared in equal amounts (20 μg) of protein. The samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto 0.2 μm nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membranes were blocked with 5% skim milk solution for 1 h at 4 °C and subsequently probed with primary antibodies against SRY-box transcription factor 2 (SOX2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), octamer-binding transcription factor 4 (OCT4; Santa Cruz Biotechnology), SOX17 (Cell Signaling Technology, Boston, MA, USA), forkhead box A2 (FOXA2; Cell Signaling Technology), C-X-C chemokine receptor type 4 (CXCR4; Santa Cruz Biotechnology), paired box 9 (PAX9; Santa Cruz Biotechnology), GATA-binding protein 3 (GATA3; Cell Signaling Technology), SOX9 (Cell Signaling Technology), KRT5 (Abcam, Cambridge, UK), and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology), followed by the corresponding secondary antibodies. The protein expression was determined using chemiluminescent reagents (Amersham, Buckinghamshire, UK) and analyzed using a luminescent image analyzer (Cytiva, Marlborough, MA, USA).

Immunofluorescence assay

TMSCs were fixed in 24-well plates with 4% paraformaldehyde for 30 min at room temperature and washed twice with DPBS. The cells were permeabilized and blocked with 1% bovine serum albumin for 1 h. Subsequently, the cells were incubated with primary antibodies against anti-OCT4 (Santa Cruz Biotechnology), anti-SOX2 (Santa Cruz Biotechnology), anti-SOX17 (R&D Systems, Inc. Minneapolis, MN, USA), anti-FOXA2 (Cell Signaling Technology), anti-CXCR4 (Santa Cruz Biotechnology), anti-PAX9 (Santa Cruz Biotechnology), anti-GATA3 (Cell Signaling Technology), anti-KRT5 (1:100 dilution; Abcam), and anti-FoxJ1 (Thermo Fisher Scientific), followed by the addition of Alexa Fluor 488 or 594 secondary antibodies (Thermo Fisher Scientific). To stain the phalloidin protein, we used Alexa Fluor 647-conjugated phalloidin antibody (Thermo Fisher Scientific). Nuclei were counterstained with VECTASHIELD® HardSet™ Antifade Mounting Medium with 4′,6-diamidino-2-phenylindole. Images were captured using a confocal microscope (LSM5 Pascall; Carl ZEISS, Oberkochen, Germany).

Statistical analysis

All results are presented as the mean ± standard deviation. Statistical significance was determined using the Student's *t-test* or one-way analysis of variance. Statistically significant values are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ or indicated using different alphabets. All experimental trials were repeated thrice to confirm the reproducibility of the outcomes.

Results

Basic characteristics of TMSCs

We established MSCs from tonsil tissue, referred to as TMSCs, which exhibited the typical MSC morphology of fibroblast-like spindle shapes (Fig. 2A). TMSCs expressed pluripotency-specific transcription factors, including OCT4, SOX2, and Nanog (Fig. 2B). Following the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) criteria for defining human MSCs [23], we assessed their phenotype and differentiation capacity. As illustrated in Fig. 2C, TMSCs exhibited high expression of the positive surface markers CD73 (97.62%), CD90 (97.74%), and CD105 (93.79%). In contrast, the negative surface markers CD14, CD34, and CD45 were expressed at minimal levels (each < 0.01%). We demonstrated the capability for tri-lineage mesenchymal differentiation into adipocytes, chondrocytes, and osteocytes—another crucial standard for defining MSCs. Differentiated TMSCs exhibited significantly higher levels of specific staining than that of undifferentiated cells (Fig. 2D).

Optimization of definitive endoderm (DE) differentiation in MSCs through varying concentrations of activin A and FBS

Activin A, a member of the TGF-β superfamily, facilitates DE induction at high concentrations because of its cellular mechanism, similar to Nodal signaling within the TGF-β signaling pathway [24]. Based on this, we assessed the optimal concentration of activin A and culture conditions for DE induction in TMSCs. Cells were treated with varying activin A concentrations (0, 5, 10, and 100 ng/mL) and FBS levels (0 and 10%). We observed that DE-related genes, such as SOX17, CXCR4, and goosecoid (GSC) were significantly upregulated at low activin A concentrations under serum-free conditions (Fig. 3A). In contrast, 10% FBS had no effect on DE induction, regardless of activin A concentration. As illustrated in Fig. 3B, treatment with 10 ng/mL activin A in a serum-free environment significantly increased the protein levels of SOX17 and CXCR4 compared with those in undifferentiated TMSCs (control [CTL]). Additionally, the protein levels of pluripotency-related transcription factors OCT4 and SOX2 were significantly lower in a serum-free environment than those in CTL. Immunofluorescence staining confirmed the enhanced expression of SOX17 (red), FOXA2 (green), and CXCR4 (red) in differentiation-induced TMSCs (DM) compared to that in CTL. In contrast, the expression of OCT4 (green) and

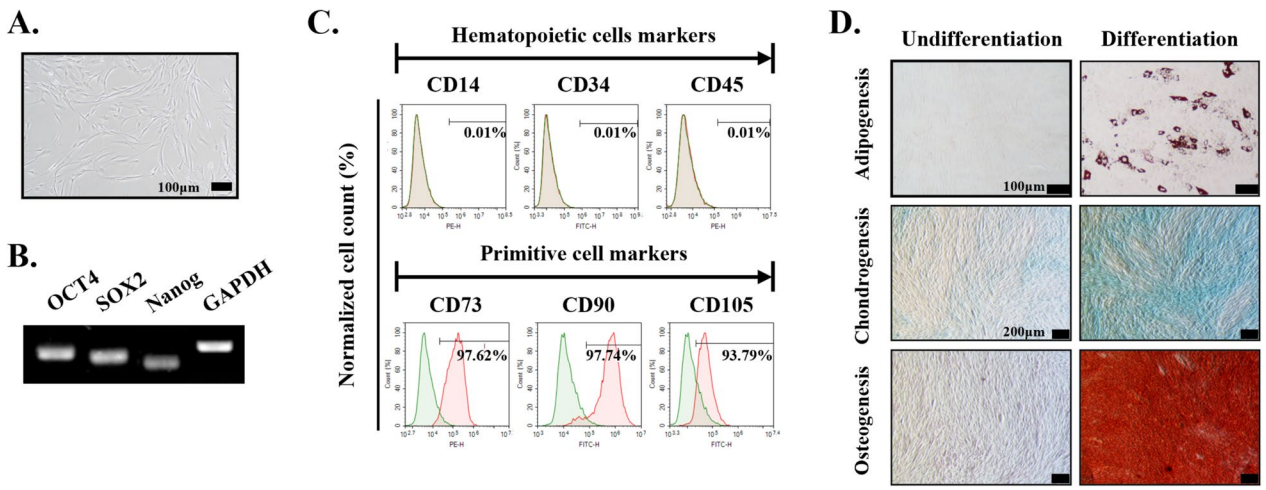


Fig. 2 Characteristics of tonsil-derived mesenchymal stem cells (TMSCs). **A** Image of cultured TMSCs at 24 h after seeding. **B** Gene expression of pluripotency-related transcription factors, such as octamer-binding transcription factor 4 (OCT4), SRY-box transcription factor 2 (SOX2), and Nanog is measured using reverse transcription-polymerase chain reaction analysis. **C** The expression of mesenchymal stem cell (MSC)-specific surface antigen markers is analyzed using flow cytometry. TMSCs are negative for hematopoietic surface markers (CD14, CD34, and CD45) and positive for MSC-specific surface markers (CD73, CD90, and CD105). **D** Multi-lineage differentiation capacity of TMSCs. Full-length blots/gels are presented in Supplementary Material 2

SOX2 (green) reduced during the transition from TMSCs to DE cells (Fig. 3C). These findings indicated that the concentration of activin A and absence of FBS are crucial for successful DE induction in TMSCs, with optimal differentiation achieved using 10 ng/mL activin A without FBS.

Induction of TMSC-derived DE cells into AFE through inhibition of BMP, TGF-β, and Wnt signaling pathways

As development progresses, respiratory organs originate from the foregut [25], and the inhibition of BMP, TGF-β, or Wnt signaling pathway is crucial to guide this process [26]. However, the appropriate combination of antagonists for each pathway and cell culture media serum concentration varies based on published studies [27]. To explore the efficient differentiation of TMSC-derived DE cells into AFE cells, we treated these cells with Noggin, a physiological inhibitor of BMP signaling; SB431542, a selective inhibitor of TGF-β/activin A/Nodal signaling; and IWP2, an inhibitor of the Wnt/β-catenin signaling pathway, adjusting FBS concentrations accordingly. Following 4 days of induction under serum-free conditions, there was a significant enhancement in the mRNA expression of AFE-specific markers, such as SOX2, PAX9, and GATA3 (Fig. 4A). Western blot analysis confirmed a significant increase in the protein levels of PAX9 and GATA3 in the DM compared to those in the CTL (Fig. 4B). Additionally, immunofluorescence staining demonstrated enhanced expression of GATA3 (green) and PAX9 (red) in differentiated cells (Fig. 4C), indicating successful induction of AFE.

Generation of airway epithelial cells through differentiation into lung progenitor cells

The SOX family and NK2 homeobox 1 (NKX2-1) are primary regulators of early cellular differentiation, specifically in the development of airway structures during the embryonic stages [28]. Their expression is enhanced in environments enriched with signaling molecules, such as bone morphogenetic protein 4 (BMP4), Wnt, and FGF [29]. To assess the successful differentiation of AFE cells into airway cell lineages, experimental groups (Fig. 5A) were designed under various conditions to monitor the expression of airway epithelial progenitor markers (SOX9 and NKX2-1) and other airway developmental markers (KRT5 and epithelial cell adhesion molecule [EpCAM]). The mRNA expression of SOX9, NKX2-1, KRT5, and EpCAM was significantly higher in the DM2 group cultured under serum-free conditions (Fig. 5B). This indicated that the environment provided in DM2 specifically facilitates the expression of genes associated with the airway epithelial lineage. Additionally, a significant increase in the protein levels of SOX9 and KRT5 (Fig. 5C) further supports the notion that inhibiting BMP signaling using Noggin significantly affects epithelial cell differentiation.

To further explore the maturation of airway epithelial cells, we modulated the Wnt and FGF signaling pathways crucial for cellular differentiation and maturation, specifically in developing airway structures. When the

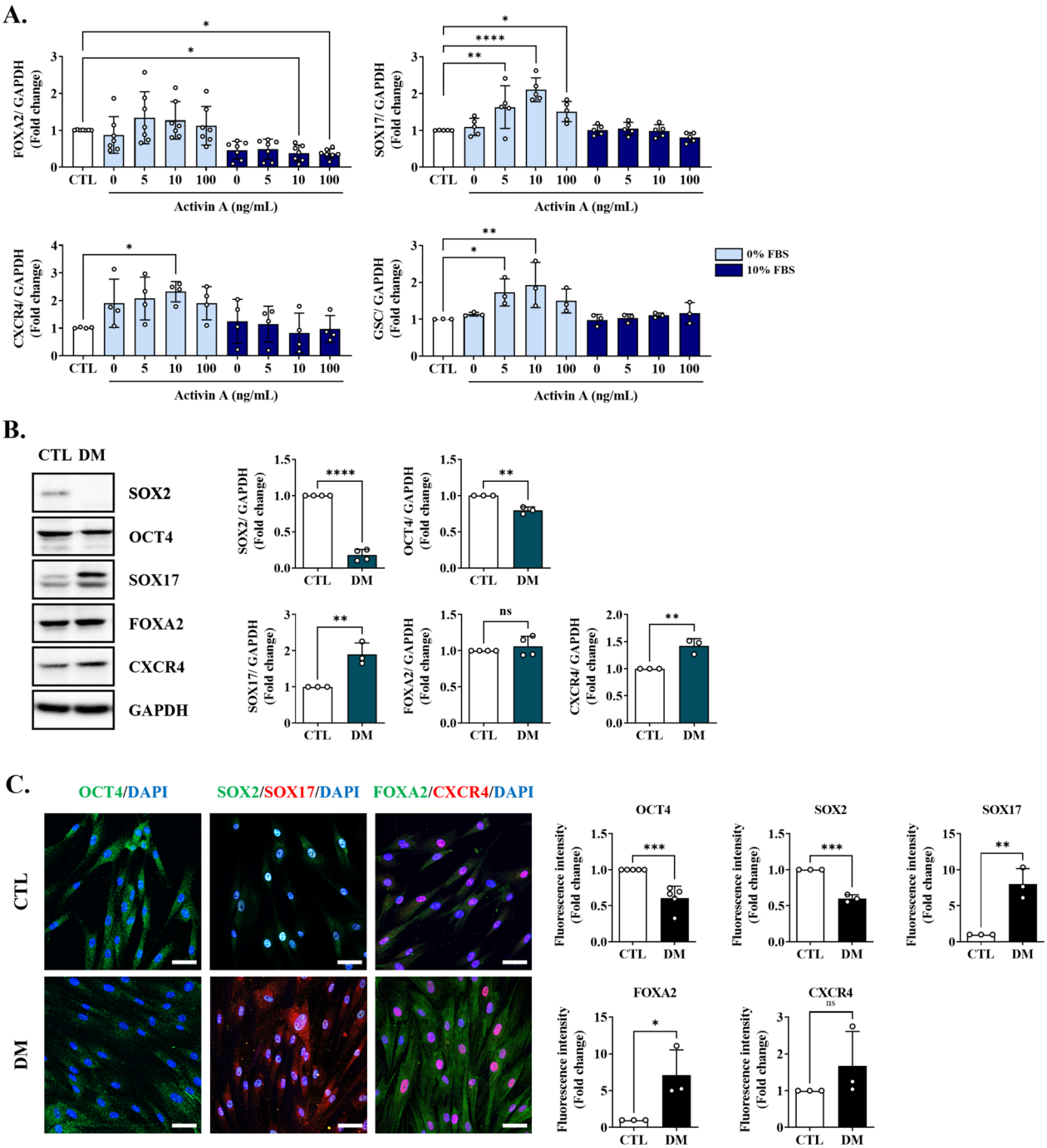


Fig. 3 A low concentration of fetal bovine serum (FBS) and activin A induces TMSCs into the definitive endoderm (DE). **A** The effects of FBS and activin A concentration on DE differentiation are assessed to quantify the DE-specific gene expression (SRY (sex determining region Y)-box 17 [SOX17], forkhead box A2 [FOXA2], C-X-C chemokine receptor type 4 [CXCR4], and goosecoid [GSC]) using quantitative real-time polymerase chain reaction. **B** The protein levels of pluripotency-related markers (SOX2 and OCT4), DE-specific markers (SOX17, FOXA2, and CXCR4), and glyceraldehyde 3-phosphate dehydrogenase are measured using western blot analysis. **C** The immunofluorescence analysis is used to confirm DE differentiation. Representative images indicate the expression of OCT4 (green), SOX2 (green), SOX17 (red), FOXA2 (green), and CXCR4 (red). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Scale bar = 50 μm. Full-length blots/gels are presented in Supplementary Material 3. The results are representative of at least three independent experimental repeats. Bars indicate the mean ± standard deviation. Statistically significant differences are denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. n.s., not significant. CTL, undifferentiated TMSCs at day 0; DM, TMSCs with differentiation media containing chemicals

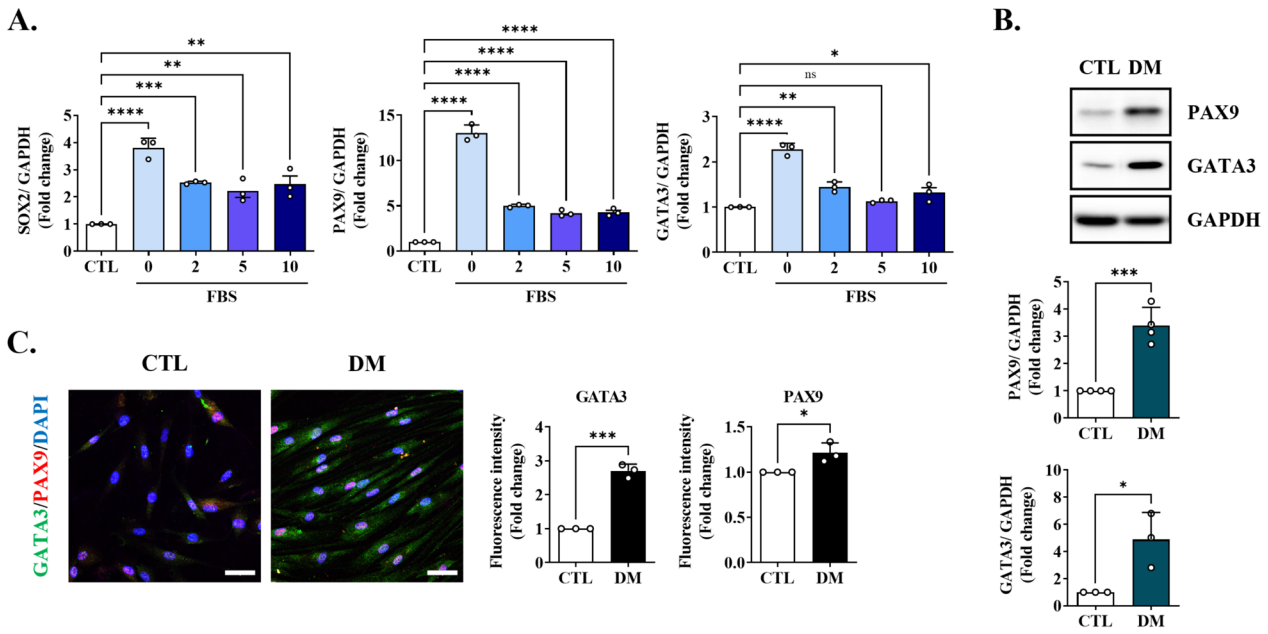


Fig. 4 Induction of TMSC-derived DE cells into anterior foregut endoderm. TMSC-derived DE cells are differentiated by inhibiting BMP, TGF-β, and Wnt signaling. **A** The expression of anterior foregut-specific markers (SOX2, paired box 9 [PAX9], and GATA-binding protein 3 [GATA3]) is measured using quantitative real-time polymerase chain reaction. **B** The protein levels of PAX9, GATA3, and glyceraldehyde 3-phosphate dehydrogenase are measured using western blot analysis. **C** Representative immunofluorescence images of two groups demonstrating GATA3 (green) and PAX9 (red). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Scale bar = 50 μm. Full-length blots/gels are presented in Supplementary Material 4. The results are representative of at least three independent experimental repeats. Bars indicate the mean ± standard deviation. The letters indicate significant differences among experimental groups (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). n.s., not significant. CTL, undifferentiated TMSCs at day 0; DM, TMSCs with differentiation media containing chemicals

cells were cultured for 14 days in the presence of FGF10, CHIR99021, transferrin, and insulin, they adopted a morphology characteristic of airway epithelial cells, displaying a round, cobblestone-like appearance distinct from the spindle-shaped morphology of undifferentiated TMSCs. Morphological transitions were evident throughout the differentiation process, with cells exhibiting an elongated shape up to step 2 and gradually adopting a shorter, pavement-like morphology from step 3 onward (Fig. 1B). These changes were consistent with the acquisition of epithelial characteristics and were corroborated by stage-specific marker expression. These phenotypic changes were consistent with epithelial lineage acquisition and were further supported by stage-specific marker expression.

To validate these morphological observations, we performed qRT-PCR and immunofluorescence analyses. qRT-PCR analysis revealed enhanced gene expression of airway cell markers, including basal cell (KRT5 and tumor protein 63), lung progenitor cell (NKX2-1), ciliated cell (FoxJ1), goblet cell (mucin 5AC), and epithelial cell (EpCAM) markers in the DM compared to those in the CTL (Fig. 5D). Immunofluorescence staining further confirmed elevated protein levels of KRT5 and FoxJ1 in the differentiated cells. In addition, to assess cytoskeletal reorganization associated with epithelial maturation, we performed phalloidin staining, a specific marker for filamentous actin (F-actin). F-actin is critical for anchoring zonula occludens proteins and plays a pivotal role in assembling tight junctions—structures essential for maintaining the barrier function of epithelial and endothelial cells, but not typically formed in MSCs. Notably, cells in the DM group exhibited a tight junction-like morphology, with F-actin showing cortical localization along the cell periphery, comparable to the staining pattern observed in human airway epithelial cells (hAECs) (Fig. 5E).

Discussion

Airway reconstruction using tissue engineering and regenerative medicine remains a significant challenge because the complete functional recovery of the airway cannot be achieved solely by implementing a biomimetic structure. To overcome this, it is essential to regenerate and implement epithelial cells that play a crucial role in the functional recovery of the airway. Similar to ESCs and iPSCs, MSCs have the potential to differentiate into specific cells through a stepwise differentiation process [30]. In this study, we demonstrated that a 4-step direct stepwise differentiation protocol induces the generation

A.

Group	Components
DM1	FGF10 + BMP4 + Retinoic acid + CHIR 99021
DM2	FGF10 + BMP4 + Retinoic acid + CHIR 99021 + Noggin

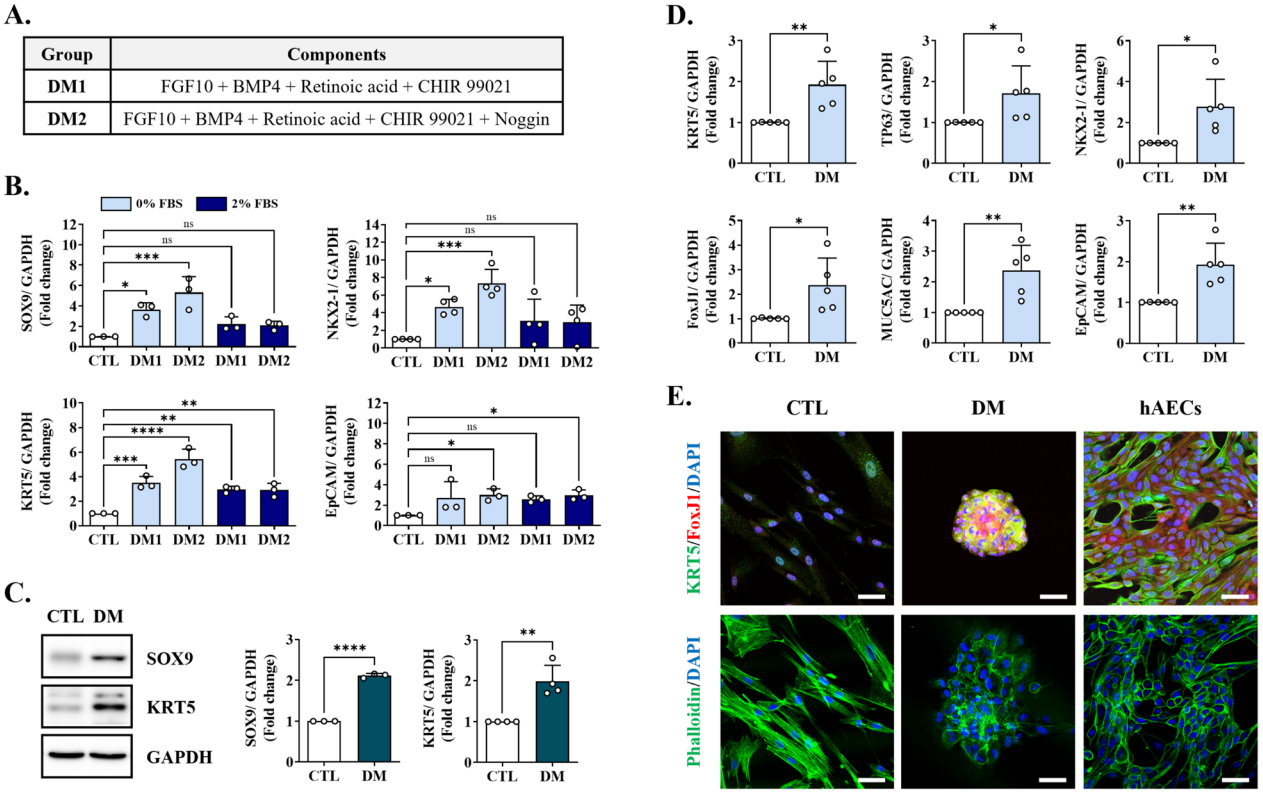


Fig. 5 Differentiation of TMSC-derived anterior foregut cells into airway epithelial cells. **A** Schematic diagram of experimental groups for differentiation into airway progenitor cells. **B** Gene expression of the airway developmental markers (SRY (sex determining region Y)-box 9 [SOX9], NK2 homeobox 1 [NKX2-1], keratin5 [KRT5], and epithelial cell adhesion molecule [EpCAM]) is quantified using quantitative real-time polymerase chain reaction. **C** Protein levels of SOX9, KRT5, and glyceraldehyde 3-phosphate dehydrogenase are measured using western blot analysis. **D** Airway progenitor cells are further differentiated into mature airway epithelial cells under the treatment of Wingless-related integration site and fibroblast growth factor signaling-related reagents. After 14 days, protein levels of airway cell markers are analyzed using western blotting. **E** Immunofluorescence staining identifies airway cell-specific markers, such as KRT5 (green), forkhead box J1 [FoxJ1] (red), and phalloidin (green). Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Scale bar = 50 μm. Full-length blots/gels are presented in Supplementary Material 5. The results are representative of at least three independent experimental repeats. Bars indicate the mean ± standard deviation. Statistically significant differences are denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. n.s., not significant. The letters indicate significant differences among experimental groups ($*p < 0.05$). CTL, undifferentiated TMSCs at day 0; DM, TMSCs with differentiation media containing chemicals; hAECs, human airway epithelial cells

of airway epithelial cells from TMSCs. This process involves a combination of specific factors, including activators, inhibitors, and culture medium supplements, to effectively modulate human airway organogenesis.

In addition to demonstrating the feasibility of airway reconstruction using a stepwise differentiation strategy, our study highlights the potential of TMSCs as a clinically advantageous cell source for airway regeneration. Unlike pluripotent stem cells, MSCs—including TMSCs—offer low immunogenicity and no risk of tumorigenicity, whereas also being easily obtainable from adult tissues such as discarded tonsils [12]. Notably, TMSCs exhibit a high proliferative capacity, enabling large-scale expansion, and have demonstrated multipotent differentiation potential under defined conditions [15–18]. In this study, we successfully guided TMSCs toward airway epithelial-like cells using a stage-specific differentiation protocol, underscoring their developmental plasticity beyond mesodermal lineages. These features support the feasibility of TMSCs as a scalable, safe, and potentially immunologically compatible source of airway epithelial cells for tissue engineering and future therapeutic applications.

The airway epithelium, which is the first structure generated during lung development, arises from DE, the progenitor stage of the entire endodermal organ system [31]. Studies using ESCs or iPSCs have emphasized the significance of using high concentrations of activin A (100 ng/mL), a member of the TGF-β superfamily, to induce the differentiation of DE cells in vitro [32]. Interestingly, TMSCs can be effectively induced into DE cells with low

levels of activin A, with only 10% of the concentration required for ESCs or iPSCs. This aligns with observations in MSCs from various tissues, where activin A concentrations < 10 ng/mL were sufficient to generate DE. Specifically, in human A-MSCs and bone marrow-derived MSCs, the expression of DE-related genes FOXA2 and SOX17 was highest in the presence of 5 ng/mL activin A for a 5 days treatment period [22, 33]. Although the detailed reason for this remains unclear, activin A secreted from human umbilical cord-derived MSCs induced neuronal development and neurite growth in neural stem cells from the subventricular zone [34]. This indicates that MSCs may inherently produce activin A during the induction process, unlike ESCs or iPSCs.

After DE generation, anterior–posterior patterning occurs to specify distinct tissues, such as the lungs, thyroid, and trachea. This patterning is essential for early foregut organ development and requires signals from the mesoderm surrounding the anterior foregut. Primary signaling pathways involved in this process include BMP, Wnt, FGF, RA, and Nodal/TGF-β, which orchestrate the complex interactions and signaling events necessary to differentiate and develop specific tissues from the foregut [35]. Treatment with Noggin/SB431542, an inhibitor of BMP and TGF-β signaling, induced the upregulation of SOX2 expression, the primary transcription factor of anterior–posterior patterning, using ESCs or iPSCs [26, 36]. Antagonizing endogenous Wnt signaling is essential for facilitating the development of anterior fate [37]. Therefore, we selected agents that inhibit BMP and Nodal/TGF-β signaling pathways for the induction of AFE cells and additionally included an inhibitor of the Wnt signaling pathway. These combined reagents successfully induced DE-derived TMSCs into AFE cells, resulting in the enhanced expression of AFE markers, such as SOX2, PAX9, and GATA3. Specifically, the enhancement of PAX9 expression at this stage indicates its potential for organizing epithelial cells. Consistent with our findings, PAX9 + AFE cells from mouse and human PSCs can effectively generate epithelial structures, including pseudostratified columnar epithelium, which lines the respiratory airways [38].

This study revealed the significance of optimizing serum concentration at each stage of stepwise differentiation. Serum, a well-known supplement for cell culture, is used to maintain stable cell survival and growth and plays a role in controlling the differentiation of various cell types, including hepatic and epithelial cells [39]. We observed that the early steps of stepwise differentiation up to the anterior foregut endodermal lineage exhibited the highest differentiation efficacy in the serum-free culture compared to cells cultured in the presence of serum. In contrast, the later stages required lower serum concentrations. These findings aligned with previous studies in which lowering the serum concentration to < 0.5% enhanced the maximal efficiency of endodermal lineage differentiation, such as DE or AFE formation, for ESCs [24, 32, 40]. Additionally, a formulation of 0.5% FBS and 5 ng/mL activin A can induce the differentiation of human A-MSCs toward DE [22].

The FGF and Wnt signaling pathways facilitated the differentiation of airway epithelial cells within TMSC-derived AFE cells. Numerous FGF ligands regulate organogenesis in multiple organ systems and participate in various biological processes. In this study, FGF10, crucial during lung morphogenesis, regulates proximal–distal patterning for branching [41]. FGF10 is highly expressed in the proximal airways, such as the trachea and primary bronchi [42, 43], and it enhances the regeneration of alveolar epithelial cells from basal cells in cooperation with fibroblast growth factor receptor 2b [44]. The DM2 group was effectively induced to differentiate into airway progenitor cells by treatment with FGF10 at concentrations double those in the DM1 group. This concentration is equivalent to twice the amount typically used in studies involving the differentiation of airway epithelial cells from iPSCs at the same stage [45]. Notably, our study offers valuable insights by identifying the optimal concentration of FGF10 that is specifically tailored for TMSCs, with higher concentrations yielding more effective differentiation. Additionally, BMP and Wnt signaling, in addition to FGF ligands, are required for the induction of NKX2-1 expression to generate lung and airway progenitor cells in mouse ESCs [46]. Consistent with these findings, we observed that treatment with the Wnt activator, CHIR99021, was necessary for the differentiation of airway epithelial and progenitor cells, as evidenced by the upregulation of NKX2-1, a lung-specific transcription factor. In this context, we selected BMP4 as the BMP family ligand based on its well-established role in early lung development. BMP4 promotes respiratory lineage specification by inducing NKX2-1 expression and repressing SOX2 in the ventral foregut endoderm [47, 48]. Several studies using ESCs or iPSCs have validated the use of BMP4 in combination with Wnt activators for efficiently inducing NKX2-1^{+} lung progenitors [49]. Although other BMP ligands such as BMP2 or BMP7 have broader roles in mesoderm and endoderm development, BMP4 remains the most specific and widely applied inducer for airway lineage commitment. This treatment facilitated the expression of proximal cell-positive markers indicative of ciliogenesis and phalloidin. However, significant ciliation was not observed in the air–liquid interface (ALI) culture on collagen-coated inserts. A few reports have emphasized the significance of standardizing optimized media for proliferation and

differentiation during ALI culture [50, 51]. Because ciliation depends on selecting appropriate media for each cell type or donor, further studies are necessary to assess suitable culture conditions for MSCs.

Airway reconstruction remains a formidable challenge in the current clinical setting because of the complexity of replicating both the structural and functional aspects of the native airway. In this context, the application of a stepwise differentiation strategy presents a promising alternative for generating functional airway epithelial cells in vitro. By recapitulating key stages of embryonic development through the sequential modulation of signaling pathways such as Activin/Nodal, BMP, Wnt, and FGF, this approach enables controlled lineage specification and has been widely applied in pluripotent stem cell studies [8, 49]. Our study demonstrates that this strategy is also applicable to adult MSCs, specifically TMSCs, offering a safer and more immunologically compatible source for regenerative airway therapies.

However, several limitations must be addressed prior to clinical translation. The stepwise differentiation process is technically demanding and time-intensive, requiring precise control of signaling cues and timing at each stage. Although we achieved stage-specific molecular and morphological transitions, full functional maturation—such as robust ciliogenesis and epithelial barrier formation—remains incomplete under current conditions. Additionally, donor-to-donor variability among TMSCs may affect the reproducibility and consistency of differentiation outcomes [52]. Future studies should focus on optimizing terminal differentiation efficiency and establishing standardized, reproducible protocols to ensure scalability and clinical applicability of this approach.

Conclusions

We developed TMSC-derived airway epithelial cells by mimicking stepwise differentiation along the known lung developmental pathways. Additionally, we confirmed that MSCs induced airway epithelial production through a combination of compounds suitable for each stage. Our results demonstrated that TMSC-derived airway epithelial cells can be an alternative source for the restoration of respiratory defects.

Abbreviations

AFE	Anterior foregut endoderm
ALI	Air-liquid interface
BMP4	Bone morphogenetic protein 4
Control [CTL]	Undifferentiated TMSCs
CXCR4	C-X-C chemokine receptor type 4
DE	Definitive endoderm
DMEM-LG	Dulbecco's modified Eagle medium containing low glucose (1000 mg/L)
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
EpCAM	Epithelial cell adhesion molecule
ESCs	Embryonic stem cells
F-actin	Filamentous actin
FBS	Fetal bovine serum
FOXA2	Forkhead box A2
GATA3	GATA-binding protein 3
hAECs	Human airway epithelial cells
iPSCs	Induced pluripotent stem cells
ISCT	International society for cellular therapy
MSCs	Mesenchymal stem cells
NKX2-1	NK2 homeobox 1
OCT4	Octamer-binding transcription factor 4
PAX9	Paired box 9
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Retinoic acid
RT-PCR	Reverse transcription-PCR
SOX2	SRY-box transcription factor 2
TMSCs	Tonsil-derived MSCs

Supplementary Information

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Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.

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The authors declare that they have not use AI-generated work in this manuscript.

Author contributions

Conceptualization, H.Y.K. (Ha Yeong Kim); Investigation, H.Y.K. and J.Y. (Jiin Yang); Writing—Original Draft, H.Y.K.; Writing—Review & Editing, H.S.K. (Han Su Kim) and S. Y. J. (Soo Yeon Jung); Funding Acquisition, S. Y. J., H.Y.K., and H.S.K.; and Supervision, S. Y. J. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

TMSCs were isolated from the tonsil tissues of patients under the age of 10 who underwent tonsillectomy, with informed written consent obtained from their legal guardians. The protocol was approved by the Institutional Review Board (IRB) of Ewha Womans University Mokdong Hospital, Korea (Approval Number: EUMC-2019–08-030). The project, titled "Isolation of Tonsil-derived Mesenchymal Stem Cells from Tonsil Tissue and Its Characterization," received IRB approval on August 30, 2019. This approval confirms that the project adheres to the necessary ethical standards and guidelines for conducting research involving human subjects.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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