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Vagal- α 7nAChR signaling promotes lung stem cells regeneration via fibroblast growth factor 10 during lung injury repair

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Abstract

Background: Proliferation and transdifferentiation of lung stem cells (LSCs) could promote lung injury repair. The distal airways of the lung are innervated by the vagus nerve. Vagal- α 7 nicotinic acetylcholine receptor (α 7nAChR) signaling plays a key role in regulating lung infection and inflammation; however, whether this pathway could regulate LSCs remains unknown.

Methods: LSCs (Sca1⁺CD45⁻CD31⁻ cells) were isolated and characterized according to a previously published protocol. α 7nAChR knockout mice and wild-type littermates were intratracheally challenged with lipopolysaccharide (LPS) to induce lung injury. A cervical vagotomy was performed to study the regulatory effect of the vagus nerve on LSCs-mediated lung repair. α 7nAChR agonist or fibroblast growth factor 10 (FGF10) was intratracheally delivered to mice. A single-cell suspension of lung cells was analyzed by flow cytometry. Lung tissues were collected for histology, quantitative real-time polymerase chain reaction (RT-PCR), and immunohistochemistry.

Results: We found that LSCs maintained multilineage differentiation ability and transdifferentiated into alveolar epithelial type II cells (AEC2) following FGF10 stimulation in vitro. Vagotomy or α 7nAChR deficiency reduced lung Ki67⁺ LSCs expansion and hampered the resolution of LPS-induced lung injury. Vagotomy or α 7nAChR deficiency decreased lung FGF10 expression and the number of AEC2. The α 7nAChR agonist-GTS-21 reversed the reduction of FGF10 expression in the lungs, as well as the number of Ki67⁺ cells, LSCs, Ki67⁺ LSCs, and AEC2 in LPS-challenged vagotomized mice. Supplementation with FGF10 counteracted the loss of Ki67⁺ LSCs and AEC2 in LPS-challenged α 7nAChR knockout mice.

Conclusions: The vagus nerve deploys α 7nAChR to enhance LSCs proliferation and transdifferentiation and promote lung repair in an FGF10-dependent manner during LPS-induced lung injury.

Keywords: Vagus nerve, Lung stem cells (LSCs), Multipotential differentiation, Proliferation

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Background

Evidence indicates that lung resident stem cells possess a proliferative capacity in vivo, and they are activated and subsequently proliferate and differentiate into lung epithelium to effectively regenerate and repair an injured lung [1–3]. Failure to regenerate the lung epithelium contributes to poor resolution in lung diseases such as pneumonia and acute respiratory distress syndrome (ARDS), and it leads to pulmonary scarring and fibrosis [4]. Thus, the reparative capacity of lung stem cells has attracted considerable research attention in terms of both elucidating the mechanism underlying regeneration after lung injury and developing therapeutic strategies for treating injured lungs.

In the adult mouse lung, the stem cell marker (Sca1) has previously been identified as a vital marker for the isolation of candidate nonendothelial (CD31⁻), nonhematopoietic (CD45⁻) bronchioalveolar stem cells (BASCs) located at the bronchioalveolar duct junction that can self-renew over multiple passages and differentiate into airway and alveolar epithelium [1]. However, McQualter et al. reported that lung fibroblastic progenitor cells are highly enriched in the Sca1⁺CD45⁻CD31⁻ population of cells [5]. Hegab et al. revealed that lung resident Sca1⁺CD45⁻CD31⁻ cells exhibited extensive self-renewal capacity and can differentiate into lung epithelial cells (alveolar epithelial type I cells: AEC1, type II cells: AEC2, and club cells) and endothelial cells, and they even exhibited some mesenchymal ability [6]. Although there is divergence regarding their differentiation capacity, lung resident Sca1⁺CD45⁻CD31⁻ cells have been consistently found to have stem cell properties and contribute to lung regeneration [1, 5–7].

During regeneration processes, the lung stem cell niche plays a key role in initiating and coordinating self-renewal and terminal differentiation. Signals involved in the cross-talk between LSCs and the microenvironmental niche include paracrine fibroblast growth factors (FGFs), which regulate cell proliferation, differentiation, motility, and survival. In particular, FGF10, which is expressed in the adjacent distal lung mesenchyme, binds to and activates fibroblast growth factor receptor 2 (FGFR2) on epithelial progenitors to direct distal lung development, including branching morphogenesis and regeneration after damage [8–12]. FGF10 signaling could be reactivated in the adult lung after injury to regenerate the epithelium [2, 11]. The exact signal triggering the FGF10 network in the context of lung repair after LPS induced injury, however, is not well understood.

The vagal nerve, the main dominant nerve of the distal airway of the lung, which contains alveoli [13, 14], is known to be regulators of both respiratory clocks [15] and local lung immunity [16]. Our group [13, 17, 18] and other groups [19–23] have reported that vagotomy or deletion of alpha 7 nicotinic acetylcholine receptor

($\alpha 7$ nAChR), the receptor for a vagal neurotransmitter, significantly amplifies inflammatory responses and exacerbates tissue damage that occurs during infection, endotoxemia, and radiation-induced lung injury. In addition, several studies have suggested that vagal signals are involved in the proliferation, differentiation, and function of various stem cells [24–27]. However, whether they regulate lung resident stem cells during the reparative phase of lung injury has not been clarified. In this study, we investigated whether and how vagal- $\alpha 7$ nAChR signaling regulates LSCs to participate in the reparative process of lung injury.

Methods

Chemicals

Lipopolysaccharides (LPS) were obtained from *Pseudomonas aeruginosa* (L9143). Dispase II, collagenase IA, deoxyribonuclease I, penicillin/streptomycin/amphotericin B, and insulin/transferrin/selenium were purchased from Sigma-Aldrich (St Louis, MO, USA). GTS-21 dihydrochloride (DMBX-A) (ab120560), a specific $\alpha 7$ nAChR agonist, was purchased from Abcam (Cambridge, MA, USA). FGF10 was provided by Newsunmit, Shanghai, China. Anti-mouse CD16/CD32 and phycoerythrin (PE) rat anti-mouse/human CD44 monoclonal antibodies (IM7) were purchased from eBioscience (San Diego, CA, USA). Fixable viability stain 780 (FVS780), allophycocyanin (APC) rat anti-mouse CD45 (clone 30-F11), APC rat anti-mouse CD31 (clone MEC 13.3), and PE rat anti-mouse Ly-6A/E (clone D7) were obtained from BD Biosciences (San Jose, CA, USA). PE/Cyanine 7 Armenian hamster anti-mouse/rat (clone HM β 1-1) CD29, PE rat anti-mouse CD105 (clone MJ7/18), and fluorescein isothiocyanate (FITC) anti-mouse TER-119/erythroid cells (clone TER-119) were obtained from Biolegend (San Diego, CA, USA). A rabbit anti-FGFR2 antibody was purchased from Abcam (Cambridge, MA, USA).

Animals

$\alpha 7$ nAChR knockout mice ($\alpha 7$ nAChR^{-/-}, background, C57BL/6J, B6.129S7-*Chrna7*tm1Bay/J, stock no. 003232) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) [13]. Littermate wild-type mice ($\alpha 7$ nAChR^{+/+}, background, C57BL/6J, 6–8 weeks old) were used as controls. The mice were housed with free access to food and water in 12-h dark/light cycles. Anesthetization was induced by intraperitoneal injection of pentobarbital sodium (80 mg/kg). Subsequently, FGF10 (5 mg/kg) dissolved in phosphate-buffered saline (PBS) or PBS with equal volume was intratracheally delivered. Three days later, mice were intratracheally injected with 2.5 mg/kg of LPS dissolved in PBS. Mice were humanely sacrificed by lethal overdose of pentobarbital sodium on the 7th day. Animal experiments were approved by the Committees

on Animal Research of the Institut Pasteur of Shanghai, Chinese Academy of Sciences, China. The protocols were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th Edition, 2011).

Unilateral vagotomy

Cervical vagotomies were performed as described previously [28]. Briefly, a longitudinal midline incision was made in the ventral region of the neck before blunt dissection. The overlying muscles and fascia were separated until the right vagus was visible. For the vagotomy (Vx) group, the vagus was carefully stripped away from the carotid artery and was carefully cut. For the sham group, the vagus was kept intact. The wound was closed and sutured.

Animal treatments

After undergoing vagotomy, mice were intratracheally injected with either the indicated concentration (2.5 or 5 mg/kg) of LPS dissolved in PBS or an equal volume of PBS. Subsequently, GTS-21 (4 mg/kg), FGF10 (5 mg/kg), or PBS was intratracheally delivered.

Histological analysis

Mouse lung sections were stained with hematoxylin-eosin to determine the extent of injury. The lung injury was graded in a blinded manner as described previously [29]: 0 = no injury; 1 = area of lung injury less than 25%; 2 = area of lung injury between 25 and 50%; 3 = area of lung injury between 50 and 75%; and 4 = area of lung injury more than 75%. Immunohistochemistry was performed with an anti-prosurfactant protein C (proSP-C) antibody (MeckMillipore, Darmstadt, Germany) or an anti-FGF10 antibody (ABclonal, Boston, USA). Sections were covered with a DAB tetrahydrochloride solution and then were counterstained with hematoxylin. The numbers of SP-C-positive cells were counted in 6 random fields ($\times 200$), and the results were expressed as the average number of SP-C-positive cells/lung nucleated cells from 3 or more animals in each group. Scoring of FGF10 protein expression was determined by the hybrid scoring system (H-score) [30]. The intensity of the staining was classified into 4 grades: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining, and then score was multiplied by the percent of positive cells in the area. All the scores were performed in a blinded manner.

Flow cytometry

After preincubating for 15 min with anti-mouse CD16/32 antibodies, lung cells were labeled with primary or isotype control antibodies. Isotype antibodies and unstained controls were set to demonstrate specificity of

staining and to establish the criteria for target populations (for simplicity, data were not shown regarding these controls). Debris and aggregates were excluded, and live cells were analyzed by LSRFortessa (BD Biosciences, San Jose, CA, USA). Data were analyzed by FlowJo vX.0.7 software (Tree Star Inc., Ashland, OR, USA).

Isolation of lung stem cells

Single-cell suspensions of lung tissue were prepared for each experiment using 3–5 mice as described [5] with modification. In brief, 1 ml of dispase (2 U/ml) was injected through the trachea. Subsequently, the trachea was removed, and the lungs were minced and incubated in a 37 °C shaking incubator for 45 min in 2 ml of 2 μ g/ml collagenase/dispase containing 0.001% DNase. These lung suspensions were filtered through 40- μ m cell strainers, centrifuged, and depleted of red blood cells using RBC lysis buffer. Subsequently, $Scal^+CD45^-CD31^-$ cells were sorted using MoFlo Astrios (USA).

Cell culture

Sorted cells were resuspended at a concentration of 1×10^6 cells/ml in DMEM/F12 supplemented with L-glutamine/pyruvate (Invitrogen), 0.5% or 10% fetal bovine serum (FBS) (Gibco, USA), penicillin/streptomycin/amphotericin B, and insulin/transferrin/selenium. Differentiation experiments were carried out using “mesenchymal stem cell functional identification kit” (Cyagen Biosciences Inc., Santa Club, CA, USA) according to the manufacturer’s protocols.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells or homogenized lungs using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. RNA was reverse-transcribed to generate cDNA using a reverse transcriptase kit (Toyobo, Tokyo, Japan), which was followed by quantitative real-time polymerase chain reaction (RT-PCR) analysis (Toyobo, Tokyo, Japan). The primers were as follows: SP-C: 5’-GGAGCACCGAAACTCAGAA-3’ (forward), 5’-CTGGCTTATAGCCGTCAGG-3’ (reverse); club-cell specific protein (CCSP): 5’-ATGAAGATCGCCATCACAATCAC-3’ (forward); 5’-GGATGCCACATAACCAGACTCT-3’ (reverse); FGF10: 5’-TTTGGTGTCTTCGT TCCCTGT-3’ (forward); 5’-TAGCTCCGCACATG CCTTC-3’ (reverse); Ki67: 5’-ATCATTGACCGCTCCTTTAGGT-3’ (forward); 5’-GCTCGCCTTGATGGTTCCT-3’ (reverse); 18s: 5’-CGGCTACCACATCCAAGGAA-3’ (forward); and 5’CCTGTATTGT-TATTTTCGTCACCTACCT-3’ (reverse). The relative expression levels of genes were determined by the $2^{-\Delta\Delta CT}$ method [31] and were normalized to 18s.

Immunofluorescence staining

Cells were seeded on cell culture chamber slides (NEST, San Diego, CA, USA) and allowed to adhere overnight. They were fixed with 4% paraformaldehyde and then were incubated in 5% BSA and 0.1% Triton X-100 to block nonspecific binding and permeabilize the cell membrane, respectively. Slides were mounted with fluoroshield mounting medium containing DAPI (Abcam, Cambridge, MA, USA) and were viewed on a confocal microscope (FV3000, Olympus, Tokyo, Japan). An anti-mouse pro-SP-C antibody was purchased from Protein-tech. An anti-Ki-67-eFluor 570 antibody was obtained from eBioscience (San Diego, CA, USA). An anti-alpha smooth muscle actin (α -SMA) antibody was acquired from Cell Signaling Technology (Danvers, MA, USA), and an anti-CCSP antibody was acquired from Abcam (Cambridge, MA). Anti-aquaporin-5 (AQP5), CD31, and α 7nAChR antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were FITC- and cyanine 3 (Cy3)-conjugated antibodies. All staining procedures were performed with appropriate isotype controls.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). Student's *t* tests were utilized unless there were multiple comparisons, in which cases one-way analysis of variance (ANOVA) with Turkey's correction for post hoc paired comparisons was adopted. All analyses were two sided. The significance level was set at $p < 0.05$. The results are shown as the mean \pm standard deviation (SD).

Results

LSCs possess multilineage differentiation ability and transdifferentiate into AEC2 under FGF10 stimulation

LSCs (positive for Sca1 and negative for CD45 and CD31) were sorted utilizing the sorting strategy that is shown in Fig. 1a, and then, they were seeded into culture plates. We found that LSCs demonstrated trilineage differentiation abilities: osteogenesis (Fig. 1b (i)), chondrogenesis (Fig. 1b (ii)), and adipogenesis (Fig. 1b (iii)). LSCs also possessed features of mesenchymal stem cells: positive for CD29, CD44, CD105, and Sca1 and negative for CD45, CD31, Ter119, and Flk1 (Fig. 1c). Through limiting dilution analysis, we found that LSCs had self-renewal capacity (Suppl. 1A). These results were consistent with previous investigations [6]. In addition, LSCs expressed FGFR2 (Fig. 1d), the main receptor for FGF10. When treated with FGF10, LSCs elongated and aligned themselves end to end in alveolar-like shapes (Suppl. 1B). We used Ki67 as a cell proliferation marker, since it is present throughout the active phases of the cell cycle (G1, S, G2, and mitosis) but is absent from resting cells (G0

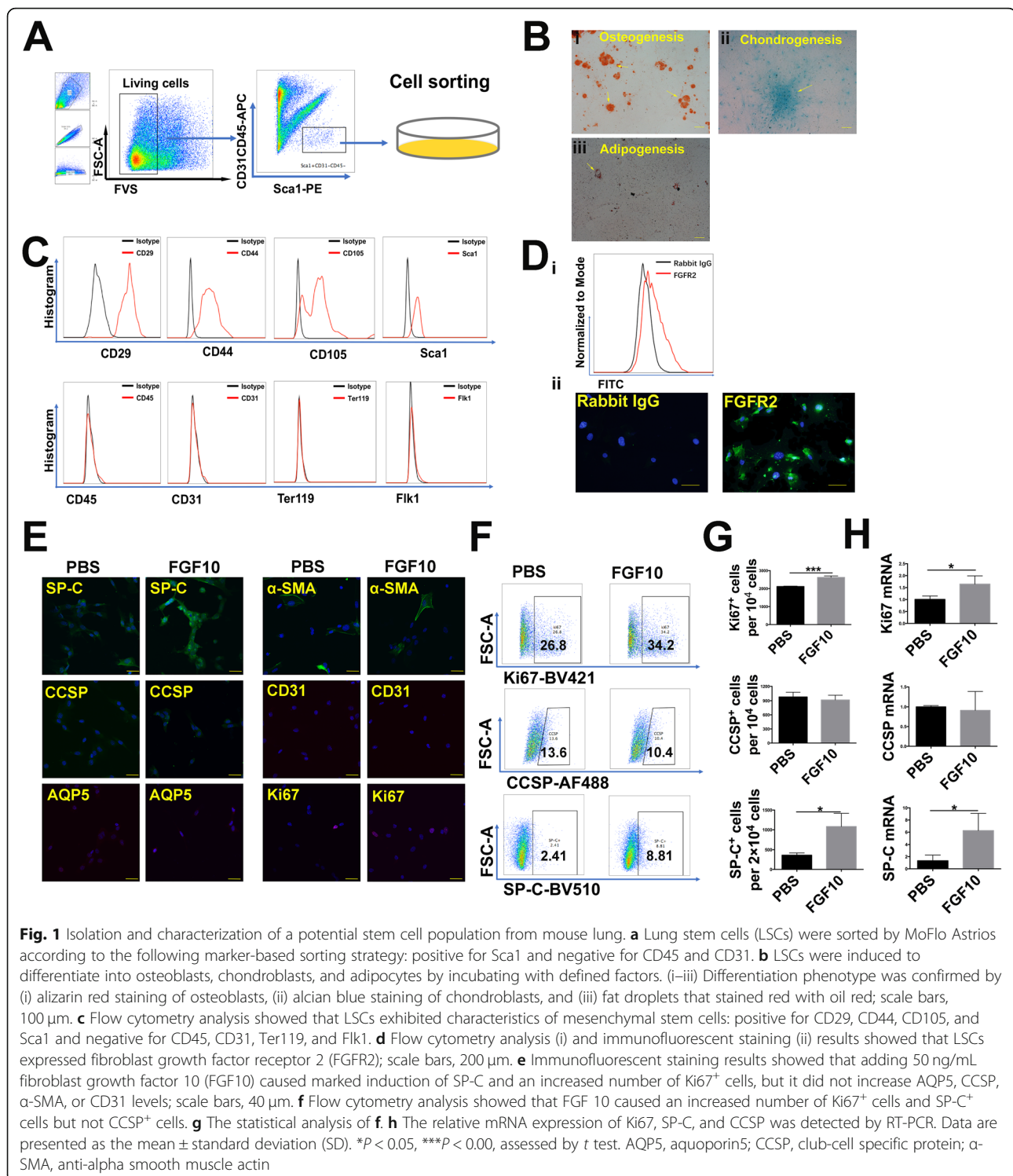
[32]. FGF10 increased the number of AEC2 (labeled by SP-C) and Ki67⁺ cells but not club cells (marked by CCSP) or AEC1 (marked by AQP5, Fig. 1e–h). The results indicated that LSCs maintained self-renewal capacity and possessed multilineage differentiation ability. FGF10 plays a vital role in mediating LSCs proliferation and transdifferentiation.

Vagus nerve does not influence the decrease in viability and proliferation of LSCs at the early phase (acute inflammation) of LPS-induced lung injury

We utilized an LPS-induced lung injury model to investigate the impact of the vagal nerve on LSCs. Mice were vagotomized 5 days before LPS challenge, as shown in Fig. 2a and b. Using the gating strategy shown in Fig. 2c, we found that there were decreased numbers of LSCs (Fig. 2d), Ki67⁺ LSCs (Fig. 2e), and Ki67⁺ cells (Fig. 2f) and a negative impact on viability (Fig. 2g) on the 1st day after LPS (5 mg/kg) challenge. Furthermore, LSCs (Fig. 2h), proliferative LSCs (Fig. 2i), and Ki67⁺ cells (Fig. 2j) were decreased on the 3rd day after LPS (5 mg/kg) challenge, but there was no decrease in cell viability of LSCs (Fig. 2k). These results indicated that LPS could cause loss of LSCs by inhibiting their proliferation at the acute phase of lung injury. However, the worsening effect of a vagotomy was not observed at the early phase of LPS (5 mg/kg)-induced lung injury.

α 7nAChR activation accelerates LSCs expansion and the reparative process at the late phase of LPS-induced lung injury

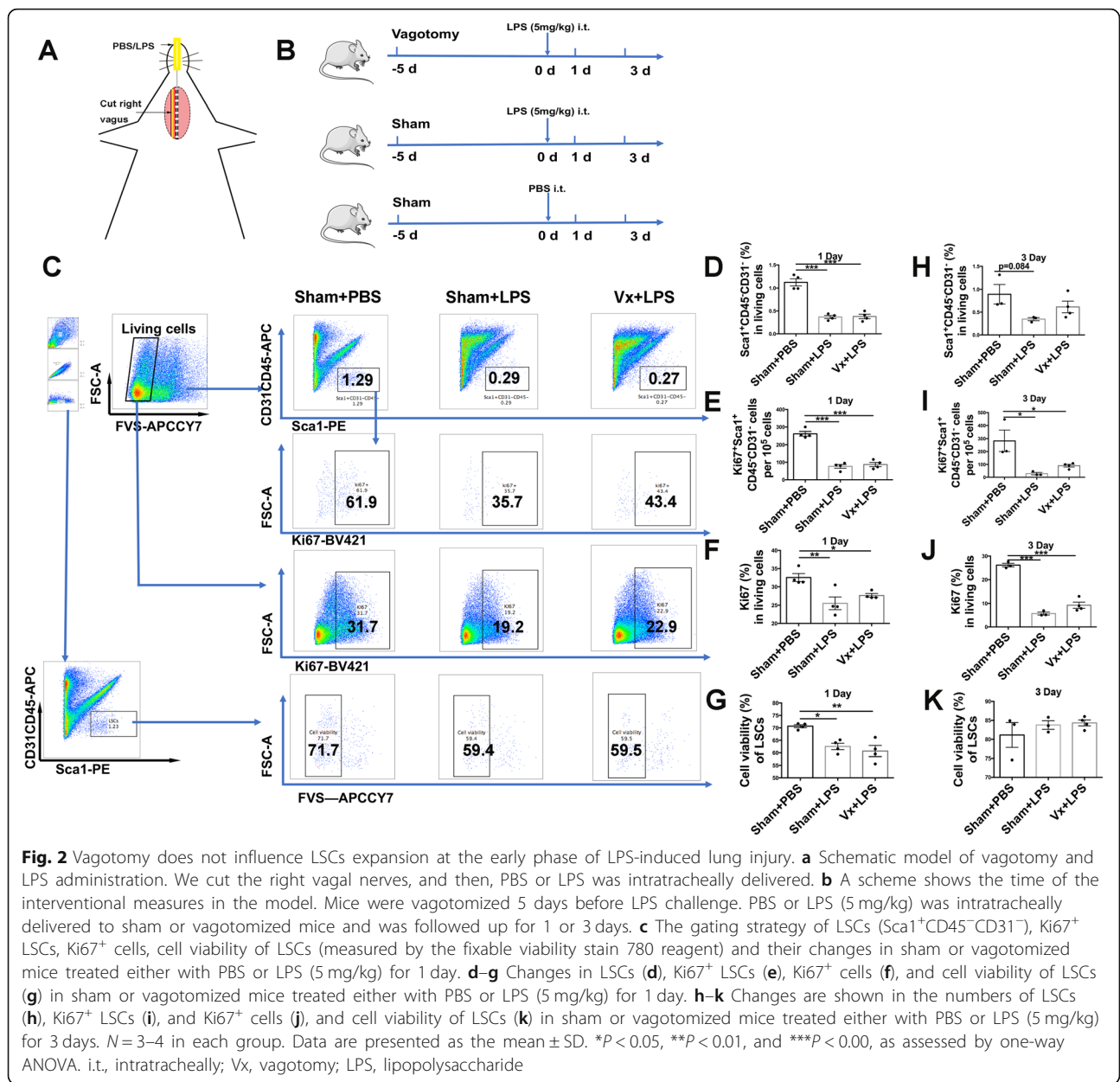
We proposed that the effect of vagal- α 7nAChR signaling on LSCs may be exerted at the late phase of LPS-induced lung injury. Because when using a dose of 5 mg/kg LPS to challenge mice, the Vx + LPS group showed high mortality after the 3rd day, we used a dose of 2.5 mg/kg LPS in the following experiments (Fig. 3a). Importantly, we observed that GTS-21 (α 7nAChR agonist) led to the expansion of LSCs and Ki67⁺ LSCs. GTS-21 treatment advanced the recovery time of LSCs from the 14th day to the 7th day after ALI (Fig. 3b) and led to a significantly increased number of Ki67⁺ LSCs on the 14th day and a slightly increased number of Ki67⁺ LSCs on the 7th day (Fig. 3c). Moreover, the degree of pathological injury appeared different in GTS-21-treated mice at later times of ALI, particularly day 3, where there was reduced exudation, decreased epithelial thickness, and a reduction in the number of inflammatory cells (Fig. 3d, e). This implied that α 7nAChR activation drove LSCs to proliferate and replenish their population and that they may also transdifferentiate into new alveolar epithelial cells for a long period after lung injury.



The vagus nerve via α 7nAChR promotes LSCs proliferation and transdifferentiation at the late (reparative) phase of LPS-induced lung injury

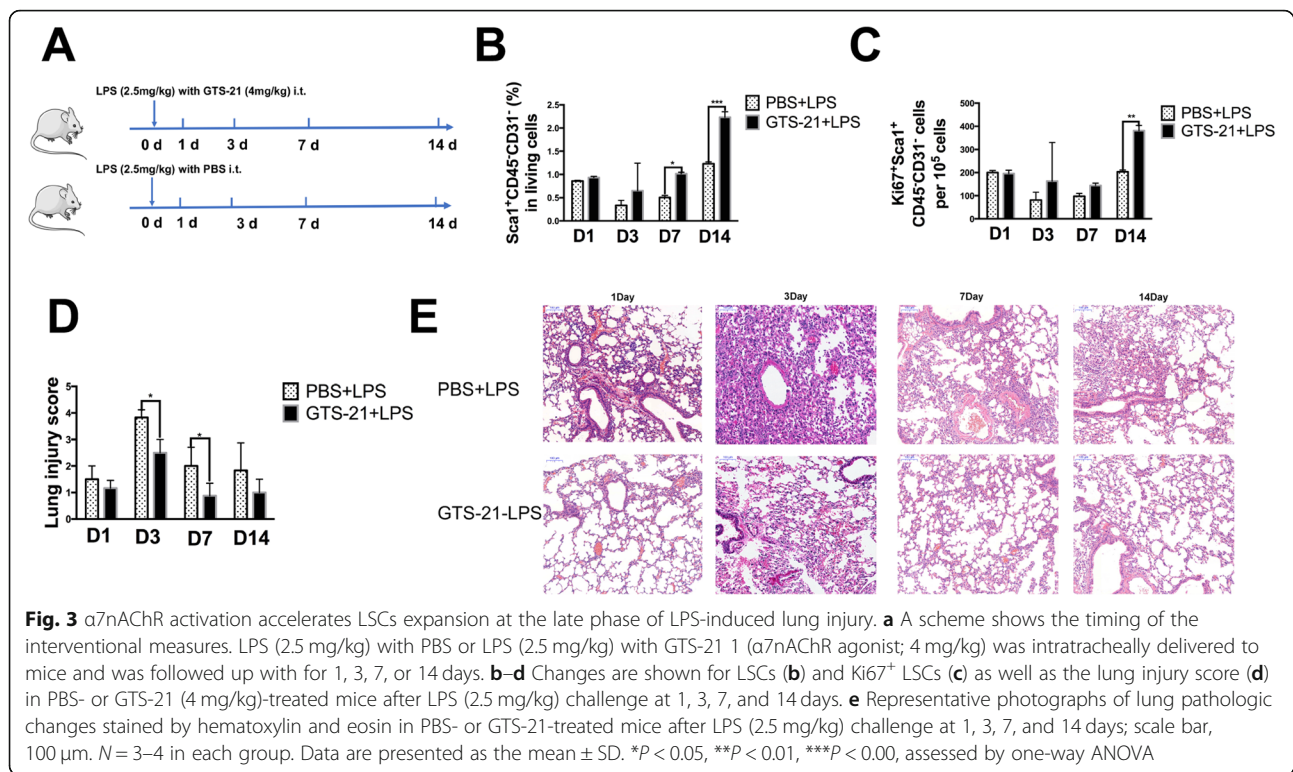
We found that vagotomy increased mortality in LPS-challenged mice, which was rescued by administration of GTS-21 (Fig. 4a). In addition, vagotomy significantly

decreased body weight by approximately 13.3% on the 12th day, delayed body weight recovery (Fig. 4b), and exacerbated lung pathological damage in LPS-induced lung injury on the 7th day (Fig. 4c, d). GTS-21 reversed vagotomy-induced body weight loss and lung injury (Fig. 4b–d). Moreover, vagotomy led to Ki67⁺ cells and LSCs loss and



inhibited Ki67⁺ LSCs without changing the cell viability of LSCs on the 7th day after LPS (2.5 mg/kg) challenge. However, activation of α7nAChR significantly resulted in the expansion of Ki67⁺ cells, LSCs, and Ki67⁺ LSCs (Fig. 4e–h), indicating that the vagus nerve promotes LSCs proliferation and enhances the reparative process via α7nAChR rather than through improving cell survival. Considering that FGF10 was found to promote the proliferation and transdifferentiation of LSCs with in vitro study, we speculated that the protective effect of vagal-α7nAChR signaling was FGF10 dependent. We found that vagotomy decreased the expression of FGF10 protein in the distal lung mesenchyme (Fig. 5a, b) and reduced FGF10 mRNA expression in the

total lungs (Fig. 5c), which was accompanied by the loss of SP-C⁺ cells (Fig. 5d, e) and reduction of SP-C mRNA (Fig. 5f) on the 7th day post LPS challenge. Activation of α7nAChR prevented the downregulation of FGF10 and accelerated SP-C⁺ cells repopulation in vagotomized mice at the reparative phase of LPS-induced lung injury (Fig. 5a–f). Collectively, these data supported the idea that the vagus nerve, via α7nAChR, promoted FGF10 expression in the distal lung mesenchyme, which then facilitated LSCs proliferation and transdifferentiation into AEC2. The LSCs expansion induced by α7nAChR activation may be explained by enhanced proliferation prevailing over the transdifferentiation process.



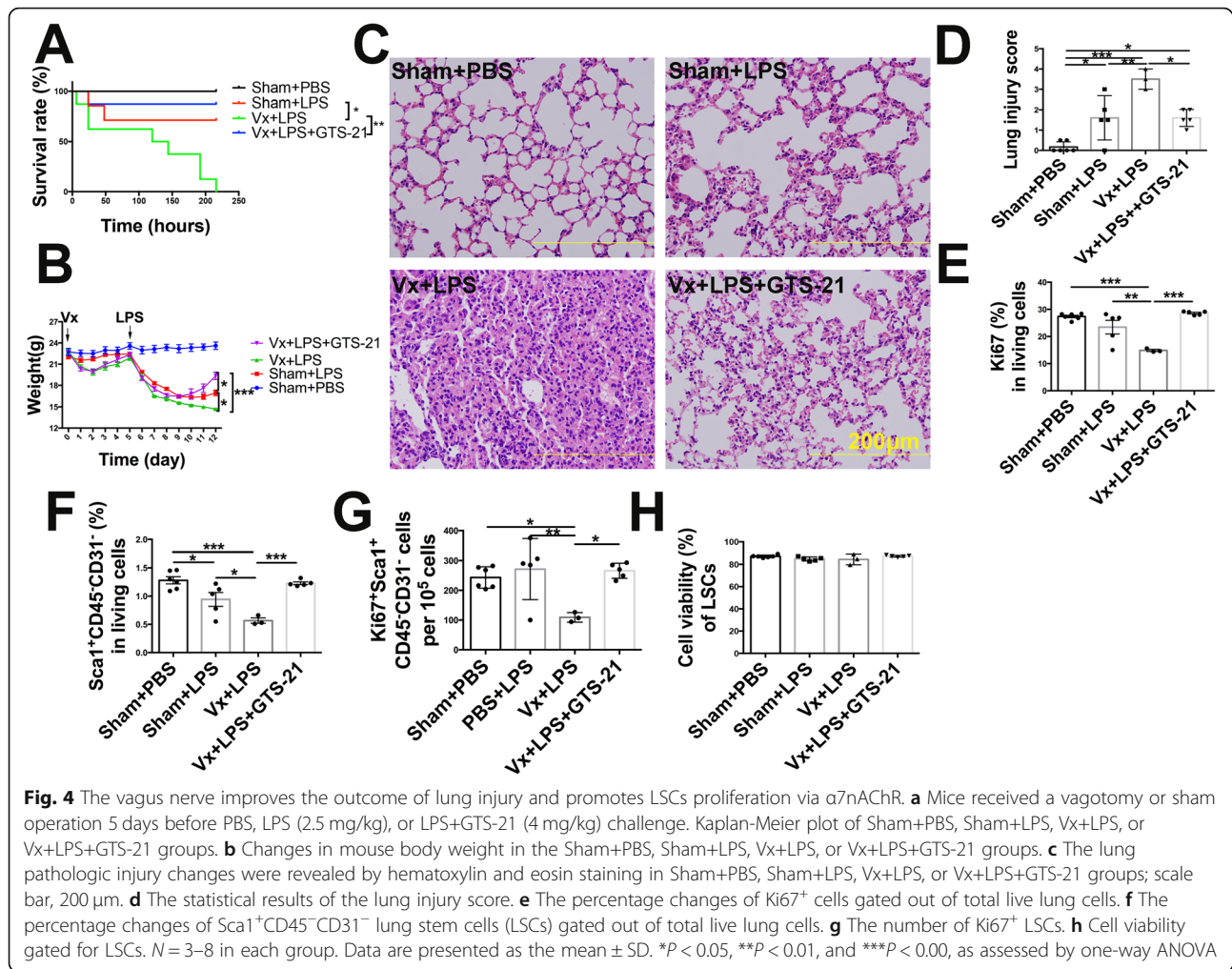
FGF10 reverses $\alpha 7nAChR$ knockout-induced loss of Ki67⁺ LSCs and reduction of AEC2

The aforementioned findings indicate that activation of $\alpha 7nAChR$ induces LSCs proliferation and transdifferentiation with activation of the FGF10 pathway engaging with vagal signals. To further confirm whether FGF10 plays an important role in this process, we used $\alpha 7nAChR$ knockout mice and designed an FGF10 rescue assay (Fig. 6a). We found that FGF10 administration partially reversed the $\alpha 7nAChR$ deletion-induced deteriorated lung damage (Fig. 6b, c). Furthermore, $\alpha 7nAChR$ ablation resulted in a significant loss of Ki67⁺ cells (Fig. 6d) and Ki67⁺ LSCs (Fig. 6e) and a downward trend of the number of LSCs (Fig. 6f) over 7-day period after LPS challenge. Importantly, $\alpha 7nAChR$ deletion-induced Ki67⁺ LSCs loss was significantly rescued by FGF10. FGF10 protein and mRNA levels were decreased in $\alpha 7nAChR$ knockout mice (Fig. 7a–c), suggesting that activation of $\alpha 7nAChR$ promotes LSCs proliferation and transdifferentiation via the FGF10 pathway. In addition, LPS-challenged $\alpha 7nAChR$ knockout lungs exhibited a decreased number of AEC2 labeled by SP-C (Fig. 7d–f). FGF10 reversed $\alpha 7nAChR$ knockout-induced loss of AEC2. These data supported the concept that LSCs deploy $\alpha 7nAChR$ -FGF10 signaling to control their fate including proliferation and transdifferentiation.

Discussion

The adult lung is a quiescent tissue under normal physiologic conditions, but it responds efficiently to injury by activating the proliferation and transdifferentiation of stem/progenitor cells to reconstitute the alveolar epithelium and promote recovery [33]. However, whether this regenerative process leads to normal or dysplastic repair is dependent on several factors, and we herein provide additional insights into this process. We clarified that the vagus nerve via $\alpha 7nAChR$ leads to LSCs proliferation and transdifferentiation and accelerates the reparative process after lung injury. LSCs proliferation and transdifferentiation during the reparative process of LPS-induced lung injury consists of transmission of the vagus nerve, lung $\alpha 7nAChR$ activation, and FGF10-dependent improved proliferation and transdifferentiation in LSCs. Moreover, vagal- $\alpha 7nAChR$ signaling was proven to be an activator of the FGF10 pathway. Thus, based on the present findings, we conclude that vagal- $\alpha 7nAChR$ signaling regulates LSCs in an FGF10-dependent manner, which leads to lung stem cells proliferation and transdifferentiation after lung injury.

Depending on the type and severity of lung injury, different cell types are involved in the reparative process after lung injury [33–35]. It has been reported in previous investigations that vagal- $\alpha 7nAChR$ signaling protects against various acute lung injuries [13, 17, 18]. To

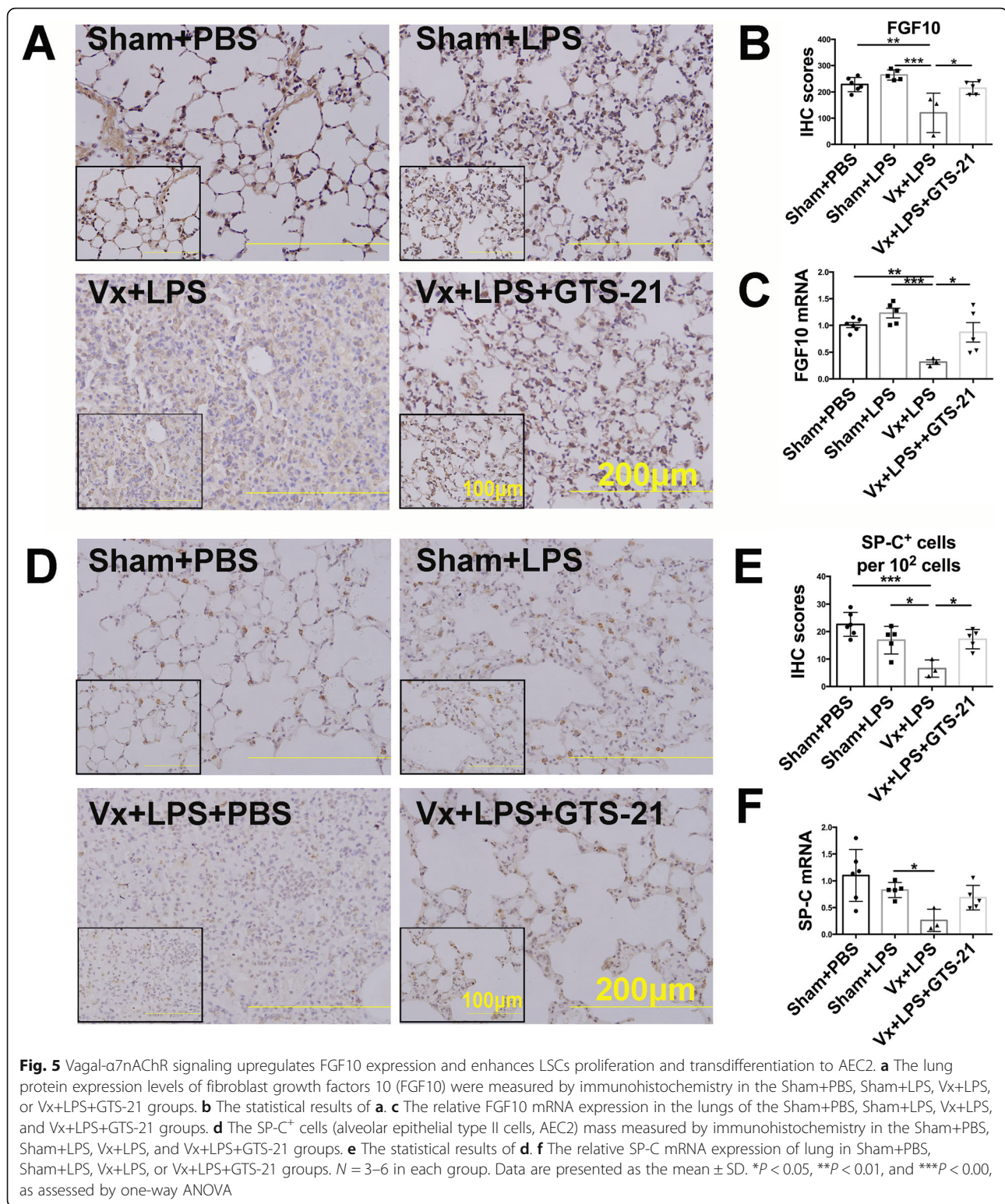


address whether this signaling affects stem cell-mediated lung repair, we comprehensively examined the changes in lung stem cell markers in LPS-challenged vagotomized mice or $\alpha 7$ nAChR-knockout mice. We found that the number of lung stem cells in Sca1⁺CD45⁻CD31⁻ populations of cells was reduced after LPS-induced lung injury, and an obvious expansion of potentially regenerative Sca1⁺CD45⁻CD31⁻ cells was observed in the mouse lung 1 to 2 weeks after LPS challenge. In addition, administration of an $\alpha 7$ nAChR agonist (GTS-21) accelerated the proliferation and expansion of Sca1⁺CD45⁻CD31⁻ cells during the reparative process after LPS-induced lung injury.

Sca1⁺CD45⁻CD31⁻ cells were originally identified as BASCs [1]. However, some studies demonstrated that Sca1⁺CD45⁻CD31⁻ cells comprise a heterogeneous population of progenitors, which are mainly lung mesenchymal progenitors [5, 10] or a population of multipotent stem cells [6]. The divergence concerning the phenotype of Sca1⁺CD45⁻CD31⁻ cells was explained by different antibodies obtained from different companies that showed

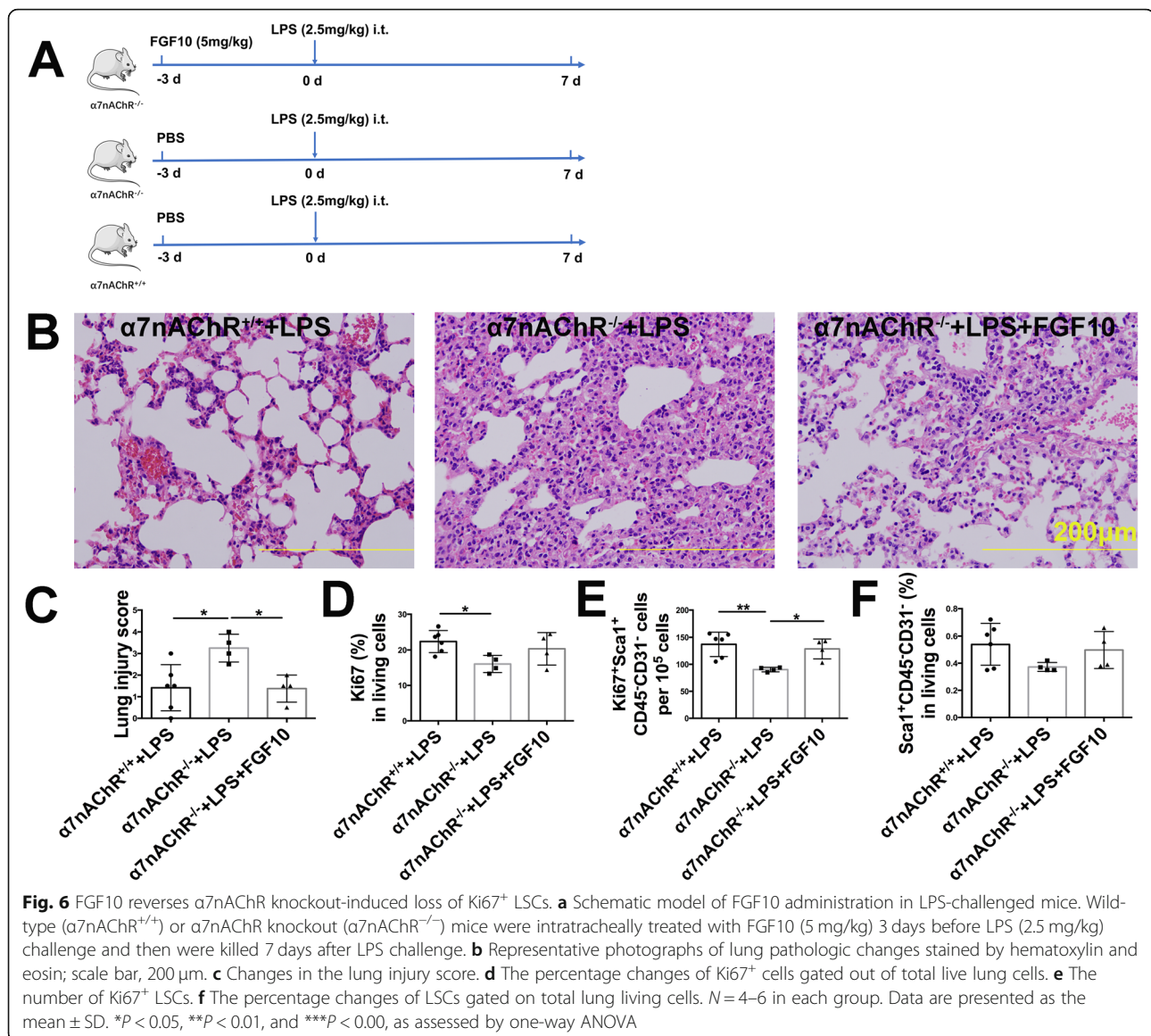
variability in the cell populations isolated according to these markers. We similarly assessed this cell population after isolation. Consistent with Hegab' study [6], we observed that LSCs possessed self-renewal capacity and possessed the characteristics of mesenchymal stem cells and lung epithelial stem cells. Hence, Sca1⁺CD45⁻CD31⁻ cells are bona fide lung stem cells and can be used to assess the lung regeneration and reparative processes. Importantly, we found that vagotomy increased mortality, delayed body weight recovery, and exacerbated lung pathological damage in LPS-induced lung injury mouse models. The application of an $\alpha 7$ nAChR agonist reversed the vagotomy-induced high mortality, body weight loss, and lung injury. Furthermore, vagotomy led to LSCs loss by inhibiting their proliferation and reduced the number of SP-C⁺ cells, but this effect could be rescued by GTS-21 administration. These data support the idea that vagal- $\alpha 7$ nAChR signaling regulates the proliferation of LSCs and promotes the regenerative process after injury.

Several lines of evidence have highlighted the importance of FGF10 in lung regeneration regulation. FGF10



overexpression has been reported to enable basal stem cells to repopulate the noncartilaginous airways [36]. Moreover, FGF10-FGFR2B signaling promoted the expansion of basal cells and alveolar epithelial regeneration

after lung injury [37]. In this study, we observed that vagotomy decreased the expression of FGF10 in the distal lung mesenchyme, which was accompanied by the loss of SP-C⁺ cells at the reparative phase after LPS



challenge. Activation of $\alpha 7nAChR$ could prevent the downregulation of FGF10 and the loss of SP-C⁺ cells. Supplementation with FGF10 could prevent $\alpha 7nAChR$ knockout-induced lung injury and $\alpha 7nAChR$ knockout-induced the loss of $Ki67^+$ LSCs and SP-C⁺ cells during LPS-induced lung injury. Previously, we also demonstrated that $\alpha 7nAChR$ deficiency could increase proinflammatory cytokines in lung injury caused by LPS challenge [17, 18] and that FGF-10 could mobilize mesenchymal stem cells and reduce lung inflammatory cytokines [38]. In addition, it has been reported that FGF10 is also expressed on a population of lipofibroblasts that are spatially associated with AEC2 cells [39]. These findings are consistent with FGF10 immunofluorescent staining in this study. Collectively, these data indicate that vagal- $\alpha 7nAChR$ signaling enhances FGF10 expression

in the lung mesenchyme and that FGF10 in turn promotes LSCs proliferation and transdifferentiation to AEC2. In addition, we also confirmed that FGF10 promotes LSCs proliferation and transdifferentiation in vitro in our study. Meanwhile, vagus nerve-induced proliferation prevails over transdifferentiation during the expansion of LSCs.

Retardation of regeneration in both the liver [40] and pancreatic islet [24] and impairment of extremity regeneration [41, 42] due to surgical denervation have been widely observed. Support for the idea that tissue regeneration is governed by neuronal signaling has been demonstrated for a few decades. However, the exact mechanisms remain unknown. This is the first study that clarifies the positive effect of vagal- $\alpha 7nAChR$ signaling at the reparative phase after lung injury. We found that the vagus nerve, via $\alpha 7nAChR$, promotes lung stem cell

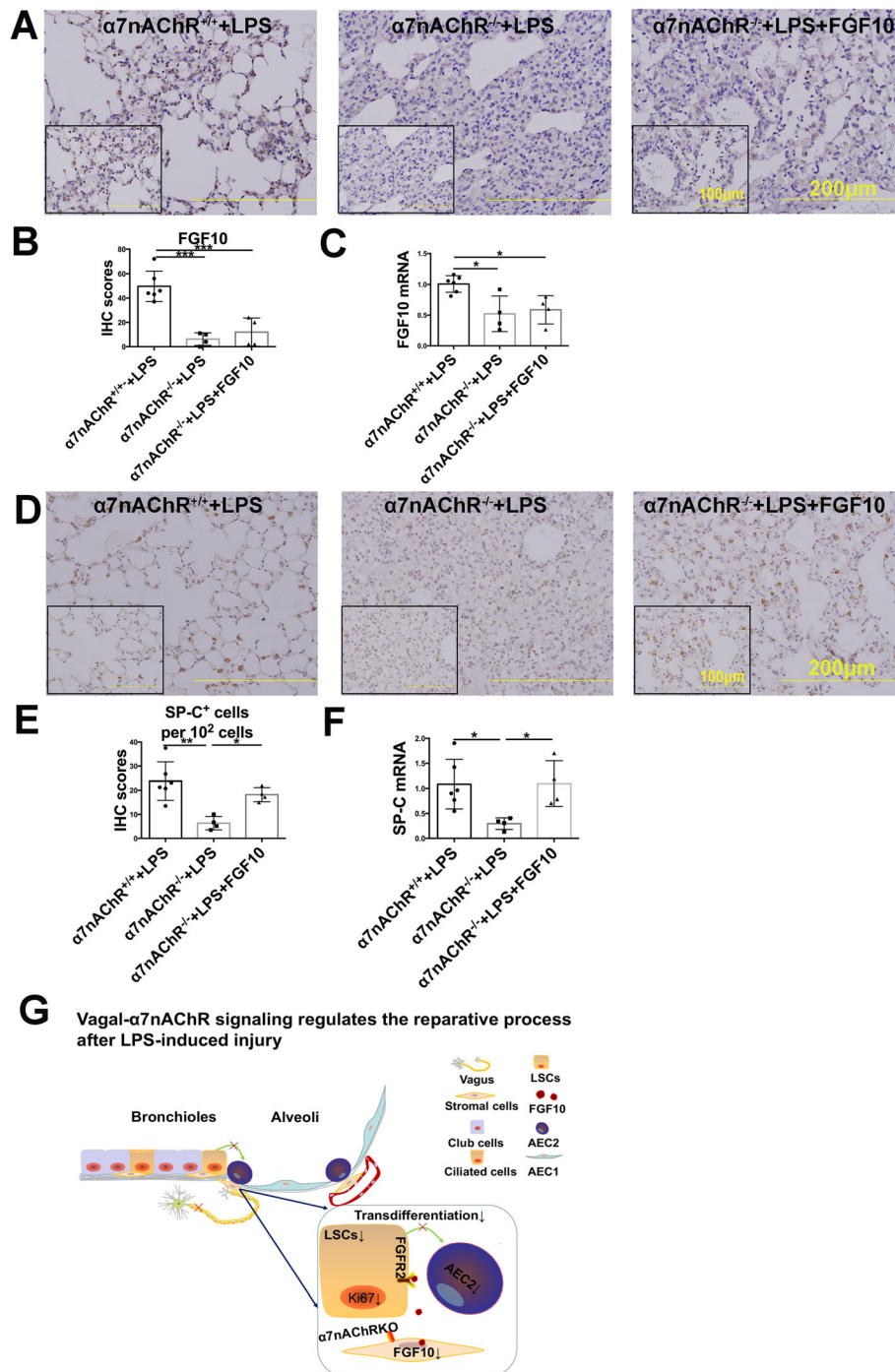


Fig. 7 FGF10 is essential for $\alpha 7nAChR$ -induced LSCs transdifferentiation to AEC2. **a** Wild-type ($\alpha 7nAChR^{+/+}$) or $\alpha 7nAChR$ knockout ($\alpha 7nAChR^{-/-}$) mice were intratracheally treated with FGF10 (5 mg/kg) 3 days before LPS (2.5 mg/kg) challenge and then were killed 7 days after LPS challenge. Lung FGF10 expression was examined by immunohistochemistry staining. **b** Changes in the FGF10 immunohistochemistry score. **c** Relative gene expression of FGF10. **d** Immunohistochemistry evaluation of SP-C⁺ cells in the lung. **e** The statistical results of the evaluation of SP-C⁺ cells. **f** The relative gene expression of SP-C. **g** Schematic model of the vagal $\alpha 7nAChR$ -mediated regenerative process after LPS-induced lung injury. *N* = 4–6 in each group. Data are presented as the mean \pm SD. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, as assessed by one-way ANOVA

proliferation and expansion in an FGF10-dependent manner. Thus, vagus stimulation and $\alpha 7$ nAChR agonist might be potent therapeutic targets for treating lung injury. The concept of vagal- $\alpha 7$ nAChR signaling governing lung stem cell proliferation may open a new avenue of research in the field of lung regeneration.

Conclusion

Vagal- $\alpha 7$ nAChR signaling promotes lung stem cell ($Sca1^+CD45^-CD31^-$) expansion and accelerates the reparative process of lung injury, and this process is FGF10 dependent. Vagus nerve $\alpha 7$ nAChR-mediated lung injury repair holds potential for the development of novel approaches to lung stem cell therapy. A schematic image showing that vagal- $\alpha 7$ nAChR signaling regulates the reparative process after lung injury is showed in Fig. 7g.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13287-020-01757-w>.

Additional file 1: Figure S1. LSCs possess self-renewal ability. A. In the LDA assay, we observed that $25.61\% \pm 5.79\%$ of the wells contained colonies. In addition, seeding of single cells that originated from secondary clones could generate tertiary clones, further confirming their self-renewal ability. Daughter colonies were always morphologically identical to primary cell colonies. Clones could be cryopreserved. And when recultured, their growth kinetics or morphology were not changed. B. When treated with FGF10, LSCs elongated and aligned themselves end to end in alveolar-like shapes within 2–3 days.

Abbreviations

LSCs: Lung stem cells; Sca1: Stem cell antigen 1; $\alpha 7$ nAChR: Alpha7 nicotinic acetylcholine receptor; LPS: Lipopolysaccharide; FGF10: Fibroblast growth factor 10; RT-PCR: Real-time polymerase chain reaction; AEC1: Alveolar epithelial type I cells; AEC2: Alveolar epithelial type II cells; ARDS: Acute respiratory distress syndrome; FGFs: Fibroblast growth factors; FGFR2: Fibroblast growth factor receptor 2; PE: Phycoerythrin; Cy3: Cyanine 3 (Cy3); FVS780: Fixable viability stain 780; APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; Vx: Vagotomy; PBS: Phosphate buffer saline; SP-C: Surfactant protein C; CCSP: Club-cell specific protein; AQP5: Aquaporin5; α -SMA: Alpha smooth muscle actin; SD: Standard deviation

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Authors' contributions

XC designed and performed the experiments and wrote the manuscript. C Zhao performed the experiments and analyzed the data. C Zhang performed the experiments and revised the manuscript. QL performed the flow cytometry and prepared the histology sample. JC performed the vagotomies. LC performed the gene identification of mice. JZ provided the reagents/materials and revised the manuscript. XS and YS conceived, designed, and revised the experiments. 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 published article.

Ethics approval and consent to participate

The protocols were approved by the Committees on Animal Research of the Institut Pasteur of Shanghai, Chinese Academy of Sciences, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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