



Cell type specific expression of Follistatin-like 1 (Fstl1) in mouse embryonic lung development

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Abstract

Follistatin like-1 (Fstl1) is a secreted glycoprotein and can be up-regulated by TGF- β 1. To better study the function of Fstl1 in lung development, we examined Fstl1 expression in the developing lung, in a cell type specific manner, using a tamoxifen inducible Fstl1-reporter mouse strain. Our results show that Fstl1 is ubiquitously expressed at saccular stage in the developing lung. At E18.5, Fstl1 expression is robust in most type of mesenchymal cells, including airway smooth muscle cells surrounding airways, vascular smooth muscle cells, endothelial cells, and vascular pericytes from blood vessel, but not PDGFR α ⁺ fibroblasts in the distal alveolar sacs. Meanwhile, relative weak and sporadic signals of Fstl1 expression are observed in epithelium, including a subgroup of club cells in proximal airways and a few type II alveolar epithelial cells in distal airways. Our data help to understand the critical role of Fstl1 in lung development and lung disease pathogenesis.

Keywords Follistatin-like 1 · Lung development · Fstl1 reporter mice · Tamoxifen induction

Introduction

Follistatin like-1 (Fstl1), initially discovered as a TGF- β 1-induced gene (Shibanuma et al. 1993), encodes a small secreted glycoprotein whose amino acid sequence has similarity with follistatin and the secreted protein acidic and rich in cysteine (SPARC) (Hambrock et al. 2004). Fstl1 is widely expressed and highly conserved in human and mouse tissues, suggesting that its functions in extracellular matrix are important and diverse. In the past decade, numerous studies have identified that Fstl1 is involved in many different biological processes in organogenesis and human disease pathogenesis (Chaly et al. 2014; Sylva et al. 2013). Loss of function experiments have unveiled an important role for Fstl1 in embryonic lung development. Conventional deficiency of *Fstl1* (*Fstl1 KO*) in mice leads to epithelium abnormality,

manifested as impaired differentiation of type I alveolar cells (AECI) and abnormal maturation of type II alveolar cells (AECII), tracheal cartilage dysplasia, manifested as discontinued rings and reduced ring number (Geng et al. 2011), and airway smooth muscle (SM) malformation, manifested as smaller SM line in trachea and discontinued SM surrounding bronchi (Liu et al. 2017). Endothelial-specific *Fstl1* deletion (*Fstl1-eKO*) in mice increases the percentage of actin-positive small pulmonary vessels leading to pulmonary vascular remodeling (Tania et al. 2017).

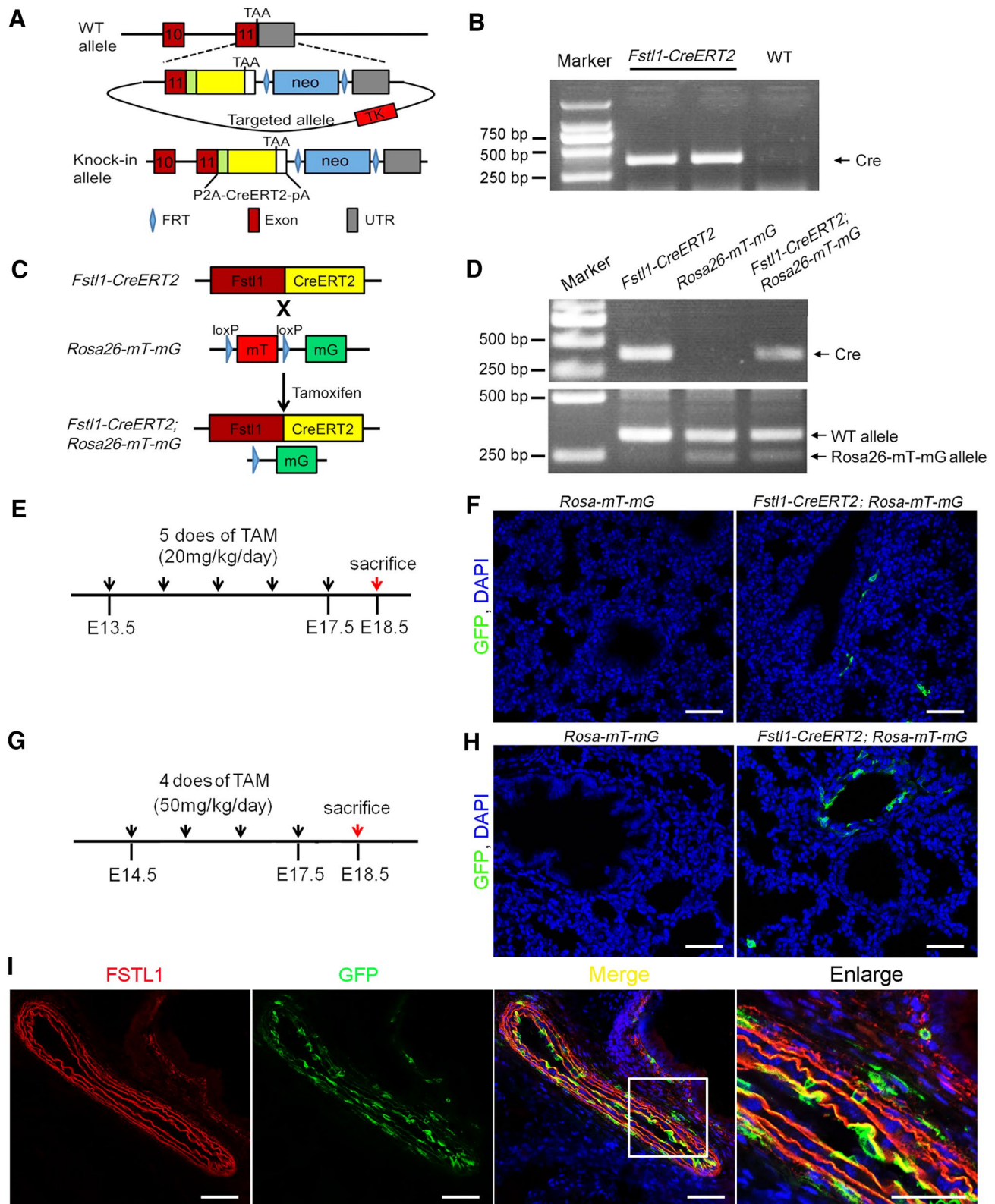
The mRNA of Fstl1 has been detected in developing lung, confirming the important role of Fstl1 in the process of lung development. Regarding the data of Fstl1 expression in mouse lung, some histological information can be found in the genepaint for E14.5 (<http://www.genepaint.org>). In addition, Adams et al. reported that Fstl1 is strongly expressed in the mesenchymal component, such as the SM layer surrounding airways, as well as walls of blood vessels using in situ hybridization (Adams et al. 2007). This mesenchymal expression pattern was also confirmed using *Fstl1-GFP* reporter mice (Sylva et al. 2011). Adams et al. (2007) also suggested *Fstl1* mRNA in goblet cells of airway epithelium by in situ hybridization. However, a detailed cell type-specific expression analysis of Fstl1 in mouse lung has not yet been conducted.

Xue Liu and Yingying Liu have contributed equally to this work.

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In order to better understand the role of *Fstl1*, we generated a *Fstl1-CreERT2* mouse line. Using *loxP-mTomato-STOP-loxP-mGFP (mT-mG)* reporter mice, we were able

to characterize the *Fstl1*-expressing lung cells during lung development. We found that in the developing lung, *Fstl1*-expressing cells overlapped with most type of mesenchymal

Fig. 1 Fstl1-expressing cells were labeled by mGFP. **a** Generation of *Fstl1-CreERT2* mice. **b** Genotypes of *Fstl1-CreERT2* and WT mice were determined by PCR. **c** Scheme of Fstl1 reporter (*Fstl1-CreERT2;Rosa26-mT-mG*) mouse generation. **d** Representative PCR products show the genotypes of the pups. Schematic representation shows low dose of TAM model: 5 doses of TAM (20 mg/kg) (**e**) and high dose of TAM: 4 doses of TAM (50 mg/kg) (**g**) to induce the recombination of *Fstl1-CreERT2;Rosa26-mT-mG* lungs. mGFP signals were detected in *Fstl1-CreERT2;Rosa26-mT-mG* lungs in both low (**f**) and high (**h**) dose of TAM models but not *Rosa26-mT-mG* lungs after TAM induction. **i** mGFP signals were co-localized with FSTL1 staining in high dose of TAM model. Boxed area was enlarged on the right. One representative example out of three embryos is shown. Scale bar 50 μ m

cells, including airway smooth muscle cells (ASMCs), vascular smooth muscle cells (VSMCs), endothelial cells, and vascular pericytes, but not PDGFR α ⁺ fibroblasts. Meanwhile, sporadic distribution of Fstl1 in Club cells and AECII cells of lung epithelium was also detected. Our data help the understanding of the detailed expression pattern of Fstl1 in lung development, which will be beneficial to the important and diverse roles of Fstl1 in lung development and different lung diseases.

Results

Generation of Fstl1-expressing reporter mouse line

We first generated a *Fstl1-CreERT2* knock-in mouse line by inserting a *P2A-CreERT2* cassette in frame followed by a *FRT-flanked* neomycin cassette before the stop codon TAA (Fig. 1a). Founder lines of mice were positive for *CreERT2* integration as detected by genomic PCR (Fig. 1b). The mouse was then crossed to a *Rosa26-mT-mG* fluorescent reporter mouse line (Muzumdar et al. 2007; Zhang et al. 2013) to generate the Fstl1 reporter mouse (*Fstl1-CreERT2; Rosa26-mT-mG*) (Fig. 1c, d). Cells with *Fstl1* expression can be easily detected by green fluorescent protein (mGFP) expression as a result of Cre mediated floxed-mTomato deletion. TAM injection can induce CreERT2-mediated recombination at the *Rosa26* locus and expression of mGFP (Muzumdar et al. 2007).

To examine the activity and specificity of Cre recombinase, we injected pregnant mice with different doses of TAM at the indicated time points and harvested E18.5 embryos for immunofluorescence analysis. As shown in Fig. 1e–h, multiple doses of TAM injections (5 doses of E13.5–E17.5 or 4 doses of E14.5–E17.5) could induce CreERT2-mediated recombination and mGFP signals in lungs of Fstl1 reporter mice, whereas, one dose of TAM (E7.5, E9.5, E14.5, or E17.5) could not (Fig. S1A–H). The mGFP signals were stronger in mice with high dose of TAM (4 doses of TAM, 50 mg/kg/day) inductions than those of low dose (5 doses of

TAM, 20 mg/kg/day) (Fig. 1e–h). In addition, we observed that multiple doses of TAM induction resulted in mGFP expression only in embryonic lung of Fstl1 reporter mice (*Fstl1-CreERT2;Rosa26-mT-mG*) by gross view. In contrast, control mice of *Rosa26-mT-mG* did not express mGFP (Fig. 1f, h). Furthermore, double immunostaining for FSTL1 and mGFP on lung sections of Fstl1 reporter mice identified the expression of mGFP in red Fstl1-positive cells (Fig. 1i), confirming the specificity of the Fstl1-driven Cre reporter system. Quantitative analysis revealed that mGFP⁺ cells could represent 76% ($75.87 \pm 12.54\%$, $n=4$) of the Fstl1⁺ cells in blood vessel, which suggested that Cre activity was not fully activated.

Fstl1 was expressed in endothelial cells, vascular smooth muscle cells, and pericytes in the pulmonary vasculature

The mRNA of *Fstl1* has been detected with high levels in walls of blood vessels (Adams et al. 2007; Liu et al. 2017; Sylva et al. 2011). To characterize the cell types with Fstl1 expression in pulmonary vasculature system, we injected the Fstl1 reporter mice with 5 doses of TAM (20 mg/kg/day, low dose), and looked at lung endothelial cells by examining co-expression of CD31, Endomucin and mGFP, and VSMCs by co-expression of α -SMA and mGFP. As expected, TAM induction from E13.5 to E17.5 resulted in mGFP expression in both CD31⁺ ($23.98 \pm 2.87\%$, $n=3$) or Endomucin-positive ($12.78 \pm 1.75\%$, $n=3$) red endothelial cells (Fig. 2a, b) and α -SMA-positive ($38.16 \pm 4.0\%$, $n=3$) red VSMCs (Fig. 2c) of E18.5 lung tissues. Finding of VSMC expression of Fstl1 is in agreement with our recent study in which Fstl1 expression in pulmonary smooth muscle cells was evidenced by co-staining of α -SMA with X-gal on adjacent lung sections from *Fstl1-LacZ* mice (Liu et al. 2017).

In addition to endothelial cells and VSMCs, we also looked at a subgroup of mesenchymal cells, pericytes, which are residing along blood vessels and marked by NG2 and PDGFR β . As shown in Fig. 2d, e, mGFP-positive pericytes ($5.26 \pm 0.96\%$ in NG2⁺ pericytes; $6.94 \pm 1.21\%$ in PDGFR β ⁺ pericytes, $n=3$) were detected in blood vessels of E18.5 Fstl1 reporter mice with TAM induction from E13.5 to 17.5. Thus, our data suggested that Fstl1 was expressed in endothelial cells, VSM cells, and pericytes in the pulmonary vasculature in lung development.

Fstl1 was expressed in ASMCs surrounding lung airways

The double immunostaining analysis for α -SMA and mGFP on lung sections of E18.5 Fstl1 reporter mice with TAM induction from E13.5 to E17.5 (low dose) or E14.5 to E17.5 (high dose) also revealed co-staining of mGFP with red

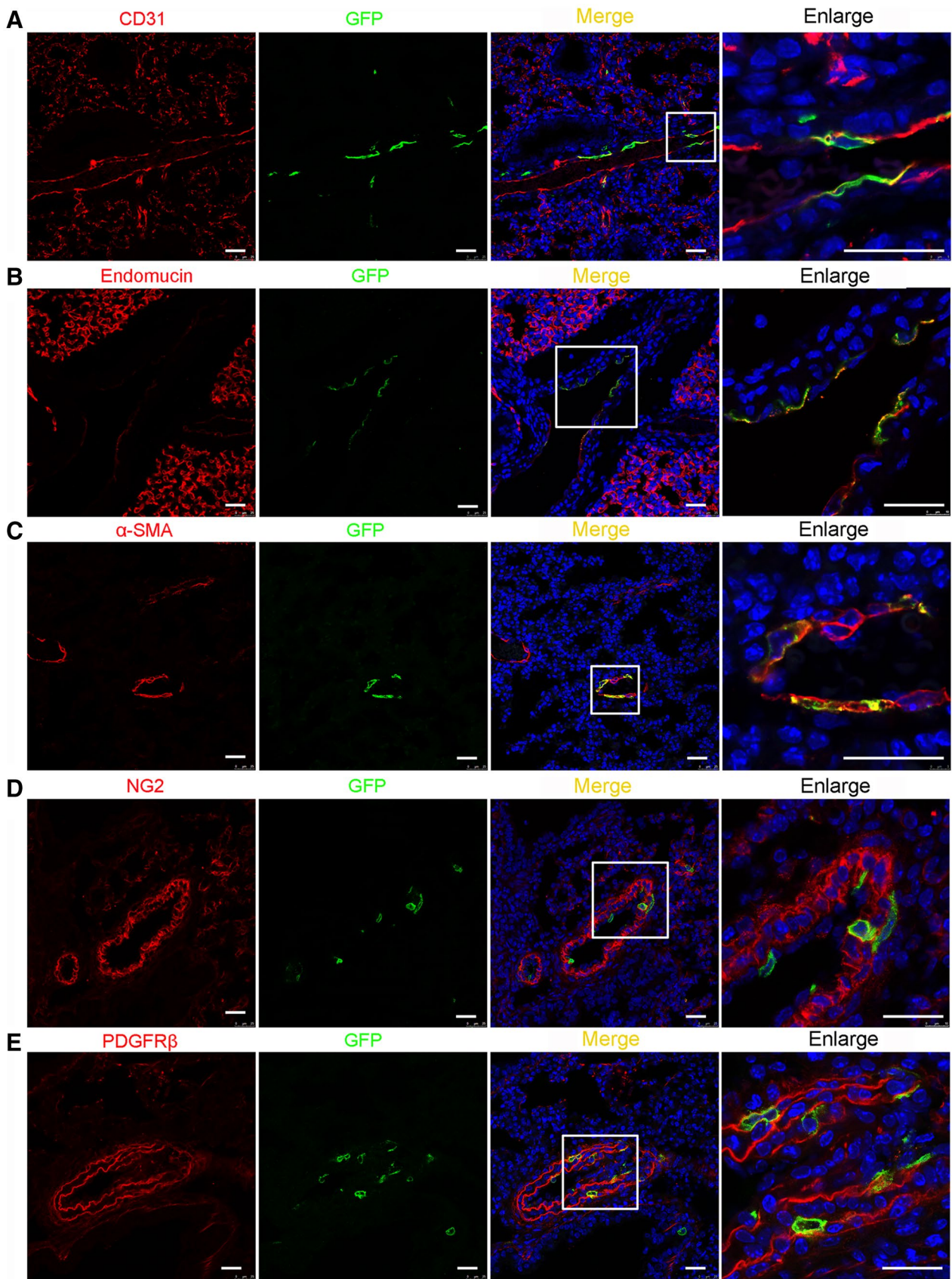


Fig. 2 *Fstl1* was expressed in endothelial cells, vascular smooth muscle cells, and pericytes in the pulmonary vasculature. Vascular endothelial cells marker, CD31 (a) and Endomucin (b), vascular SM marker, α -SMA (c), and vascular pericyte marker, NG2 (d) and PDGFR β (e) staining showed co-localization with mGFP in low dose of TAM model. One representative example out of three embryos is shown. Boxed area was enlarged on the right. Scale bar 25 μ m

α -SMA-positive ASMCs (Fig. 3), indicating the SM cell expression of *Fstl1* in developing lung airways. This is in agreement with the studies in which the mRNA of *Fstl1* was detected within the smooth muscle layer surrounding airways and *Fstl1* played an important role in ASM formation in lung development (Adams et al. 2007; Liu et al. 2017). Furthermore, the percentage of mGFP labelled cells in ASMC population was increased by TAM in a dose dependent manner (low dose, $10.74 \pm 2.83\%$; high dose, $16.28 \pm 3.54\%$, $n=3$).

***Fstl1* was expressed in a few CCSP-positive club cells in proximal airways**

Although *Fstl1* is strongly expressed in the mesenchymal component and excluded from the epithelium in the majority of organs, notable exception of the expression of *Fstl1* in goblet cells of lung airway epithelium has been reported (Adams et al. 2007). To detect whether *Fstl1* is expressed in lung epithelial cells, we performed double immunostaining for mGFP and epithelial marker E-cadherin on lung sections of E18.5 *Fstl1* reporter mice with TAM induction from E13.5 to E17.5 (low dose) or E14.5 to E17.5 (high dose). Although most epithelial cells were mGFP negative, a few mGFP + E-cadherin + epithelial cells in airways ($1.14 \pm 0.26\%$ in low dose; $1.76 \pm 0.41\%$ in high dose, $n=5$) were detected in both low and high dose of TAM treatment models (Fig. 4a). To further characterize the epithelial cell types with *Fstl1* expression, we performed co-staining for mGFP with club cell marker CCSP (Fig. 4b), ciliated cell markers β -tub IV and Foxj1 (Fig. S2 A and B), neuroendocrine cell marker CGRP (Fig. S2C), basal cell marker p63 (Fig. S2D), and goblet cell marker Mucin 5AC (Fig. S3B). Notably, only a few specific CCSP⁺ cells co-staining with mGFP were identified ($0.98 \pm 0.26\%$ in low dose; $1.48 \pm 0.20\%$ in high dose, $n=5$), no co-staining signals of mGFP with any other epithelial markers, including goblet cell marker, were detected. Specially, although we could detect some Mucin 5AC⁺ (Fig. S3A) or Alcian blue⁺ (Fig. S3C) goblet cells in trachea epithelium, we could hardly detect goblet cells in developing lung airways (Fig. S3B and D), nor co-staining signals with mGFP (Fig. S3B).

To further confirm the existence of *Fstl1*⁺ club cells, we measured the *Sgc1al* mRNA expression in sorted EpCAM⁺ mGFP⁺ epithelial cell using the single cell qRT-PCR. As expected, EpCAM⁺, mGFP⁺ cells expressed much higher

level of *Fstl1* mRNA than that of EpCAM⁺, mGFP⁻ cells (Fig. S4). Meanwhile, we did detect several EpCAM⁺ mGFP⁺ cells with mRNA expressions of both *Fstl1* and *Sgc1al* (Fig. S4), indicating the subgroup of *Fstl1*⁺ club cells. Our data suggested the *Fstl1* expression in airway epithelium and identified the expression of *Fstl1* in a subgroup of club cells of lung airway epithelium.

***Fstl1* might be expressed in a subgroup of AECII in distal alveolar sacs**

To further determine whether *Fstl1*-expressing cells contribute to epithelial cells in distal alveolar sacs, we performed co-staining for mGFP with AECII marker pro-SPC (Fig. 5a) and AECI marker T1 α (Fig. 5b). To our surprise, a couple of mGFP + pro-SPC⁺ AECII cells ($0.83 \pm 0.13\%$, $n=5$) were detected only in high dose of TAM treatment models, suggesting that *Fstl1* might express at low level in a subgroup of AECII cells in distal alveolar sacs during mouse lung development. Single cell qRT-PCR analysis confirmed a very high *Fstl1* transcription in a few *Sftpc*-expressing cells (Fig. S4). Further examination of co-staining of mGFP and T1 α revealed that AECI cells did not express *Fstl1* in neither low nor high dose of TAM models (Fig. 5b).

No detectable *Fstl1* expression in PDGFR α ⁺ fibroblasts in distal alveolar sacs

The alveolar myofibroblasts, derived from PDGFR α ⁺ fibroblasts about P2-P12 at alveolar stage of lung development (Branchfield et al. 2016), are localized in the mesenchyme at the tip of the emerging secondary septae and responsible for secondary septae formation. By deposition of elastin and collagen, the alveolar myofibroblast initiates the process of secondary septation (Chao et al. 2015). To determine whether *Fstl1* is expressed in this subgroup of mesenchymal cells, we performed double immunostaining for mGFP with alveolar myofibroblast marker PDGFR α . As shown in Fig. 6, no co-staining signals were detected in distal alveolar sacs of E18.5 *Fstl1* reporter mice with either TAM induction from E13.5 to E17.5 (low dose) or E14.5 to E17.5 (high dose). Our data indicated that *Fstl1* was not expressed in PDGFR α ⁺ alveolar myofibroblast.

Discussion

Fstl1 has been reported to be crucial during lung development, however, the cellular and molecular mechanisms underlying are largely unclear. A detailed cell type-specific expression analysis of *Fstl1* in lung needs to be conducted. The present study took advantage of genetically labeled *Fstl1*-expressing cells with mGFP and reported that *Fstl1*

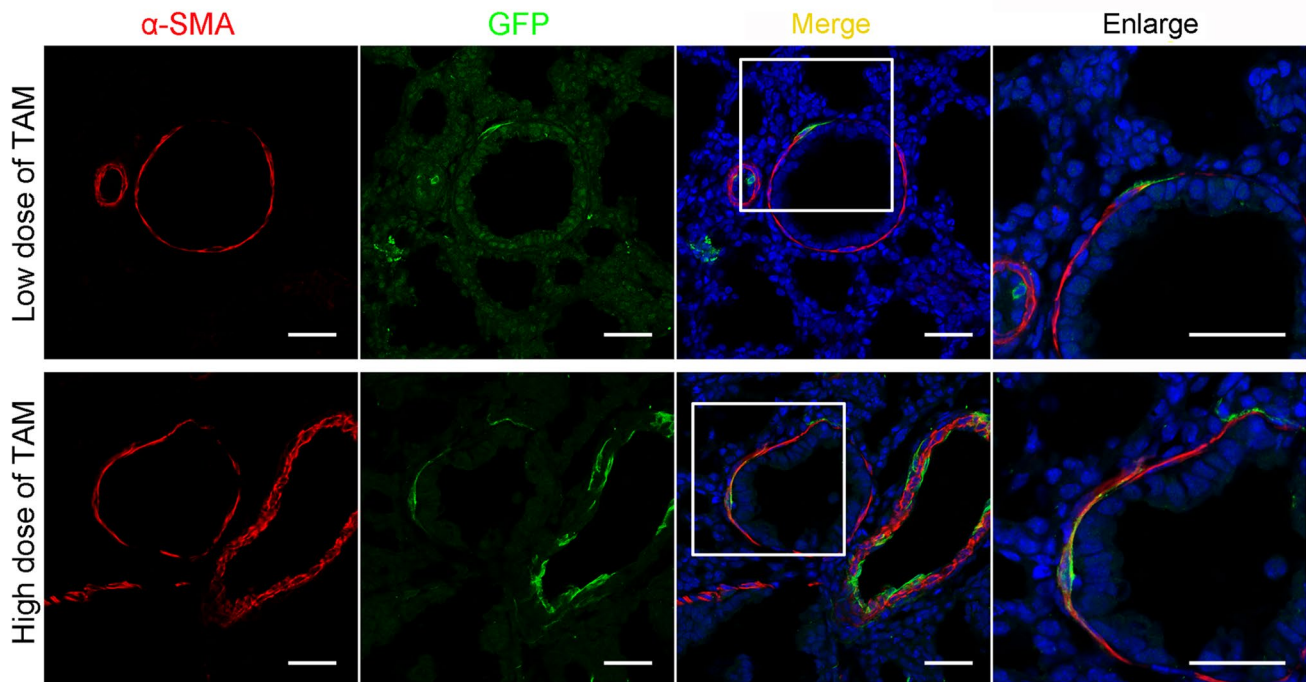


Fig. 3 Fstl1 was expressed in airway SM. Airway smooth muscle cell marker, α -SMA showed co-staining with mGFP in both low and dose of TAM models. One representative example out of three embryos is shown. Boxed area was enlarged on the right. Scale bar 50 μ m

was expressed mainly in lung mesenchyme, including VSMCs, endothelial cells and pericytes from blood vessel and ASMCs surrounding airways, but except PDGFR α ⁺ fibroblasts in distal alveolar sacs. In addition, Fstl1 expression in subgroups of club cells and AECII cells from epithelium has also been identified.

The developing lung was reported to show a generalized expression of Fstl1 at the early stage of lung development, with high levels in lung mesenchyme. Unfortunately, we could hardly detect any mGFP⁺ cells after single dose of TAM injection, partially, due to the low Cre activity. In our *Fstl1-CreERT2* mice, *P2A-CreERT2* cassette was inserted at the end of exon 11 of *Fstl1* gene which was not fully translated (<http://uswest.ensembl.org>), resulting the low Cre activity and low *GFP* transcription efficiency as well. With the help of this mouse strain, we can clearly distinguish the mGFP-labeled cells and estimate their Fstl1-expressing levels by different doses of TAM injection.

We offered the two lines of evidence to demonstrate the sporadic epithelial expression of Fstl1 in lung development. Co-immunostaining of mGFP with several epithelial cell markers revealed a subgroup of club cells and a subgroup of AECII cells with Fstl1 expression. The existence of these epithelial subgroups was further confirmed by single cell RT-PCR analysis. Our finding is consistent with the report of expression of Fstl1 in lung airway epithelium (Adams et al. 2007), but not with the conclusion that Fstl1 was expressed

in goblet cells of airway epithelium, even not in goblet cells of tracheal epithelium. Recently, accumulating data suggests that airway epithelial cells could rarely differentiate into goblet cells according to several reports and reviews during early lung development (Maeda et al. 2007; Morrisey and Hogan 2010; Rackley and Stripp 2012; Roy et al. 2011; Wan et al. 2004).

The critical functions of Fstl1 during embryonic development, especially during lung development, are frequently reported in recent years, which are closely related with its cell type-specific expression pattern. Considering the mesenchymal expression pattern of Fstl1, the impaired phenotypes of airway smooth muscle, vascular smooth muscle, and tracheal cartilage in *Fstl1 KO* mice (Liu et al. 2017; Geng et al. 2011; Sylva et al. 2011), and of pulmonary hypertension in *Fstl1 eKO* mice (Tania et al. 2017) might be due to the direct loss of function Fstl1 in corresponding cell population. Even the phenotype of spotted elastin staining and significant less elastic fiber density in E18.5 *Fstl1 KO* lung (Geng et al. 2013) might be also due to decreased elastin production from impaired endothelial cell during saccular stage (Cantor et al. 1980; Mecham et al. 1983), but the hypothesis needs to be further confirmed. Meanwhile, indirect Fstl1 functions as the results of different mesenchymal cell interaction can't be ruled out. We previously reported that Fstl1 exerts its functions on alveolar epithelial differentiation via the spatial access

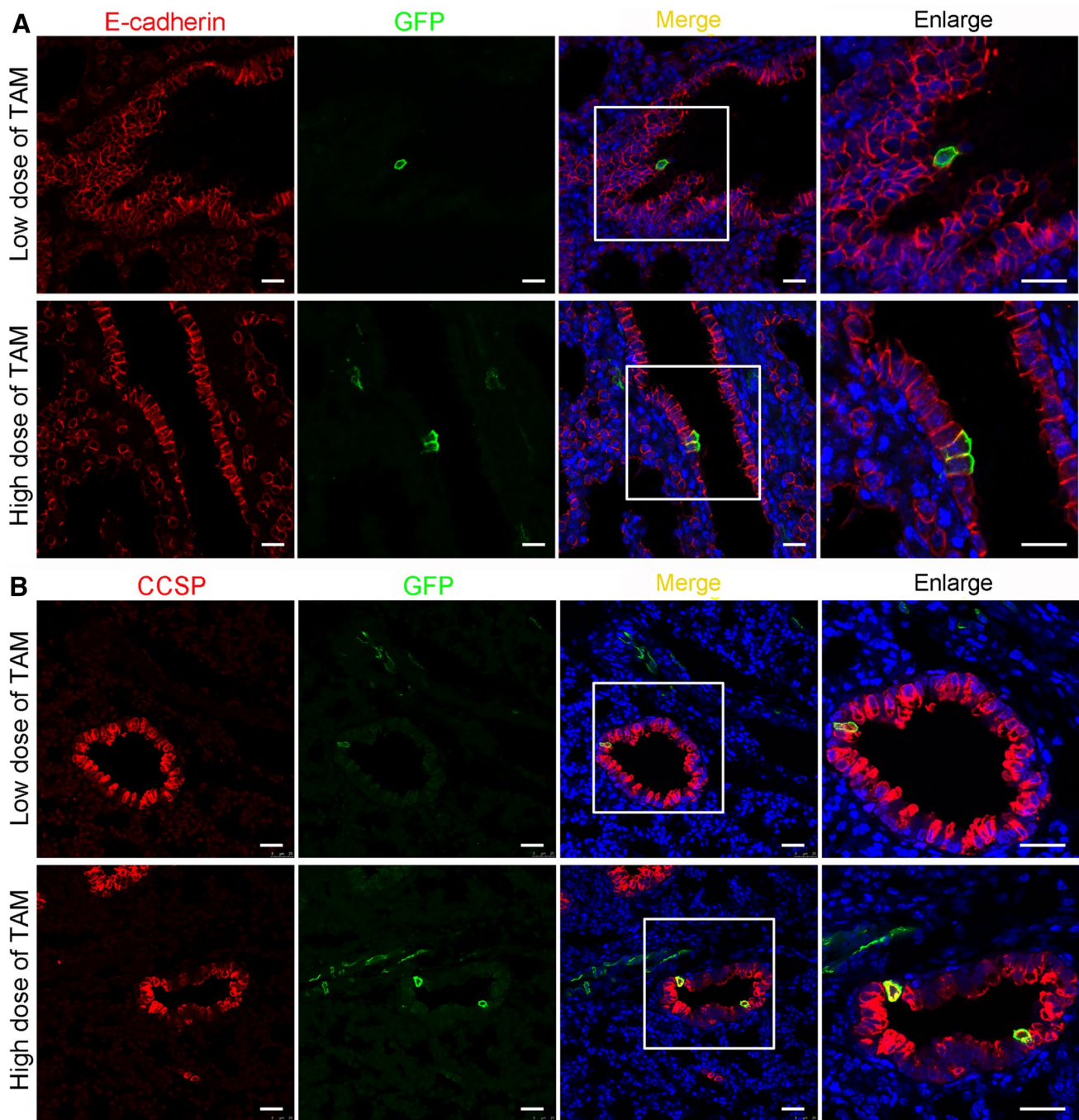


Fig. 4 Fstl1 was expressed in a few club cells. **a** E-cadherin and **b** CCSP co-staining with mGFP on lung sections of low and high dose of TAM models. Boxed area was enlarged on the right. One representative example out of three embryos is shown. Scale bar 25 μm

of mesenchymal Fstl1 to basolateral localization of BMP receptors in polarized epithelial cells (Geng et al. 2011; Li et al. 2016). This might be account for the abnormal differentiation/maturation of alveolar epithelial cells in *Fstl1 KO* lung (Geng et al. 2011; Li et al. 2016). The role of Fstl1-expressing epithelial cells in lung development remains to be revealed.

In summary, we have generated Fstl1 reporter mouse strain and identified the cell-specific expression patterns of Fstl1 in developing lung (E18.5). We find that Fstl1 is strongly expressed in most type of mesenchymal cells. Notable expression of Fstl1 in a subgroup of club cells and AECII cells from epithelium was also observed. The precise mechanisms by which Fstl1 exerts its function,

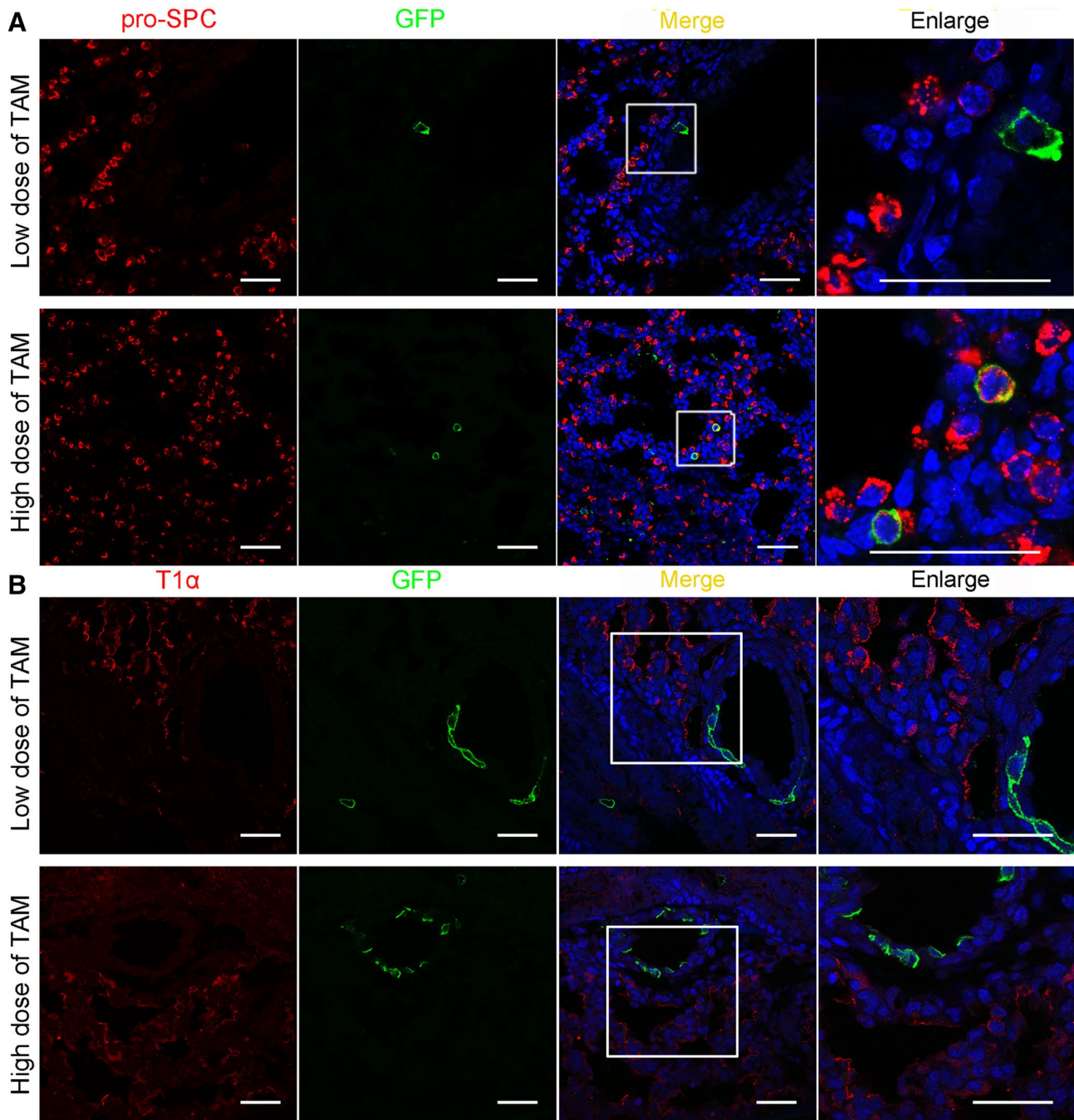


Fig. 5 mGFP signals were detected in the pro-SPC⁺ cells in high dose of TAM model. **a** Immunofluorescence for GFP and pro-SPC showed mGFP signals in pro-SPC⁺ cells in high dose of TAM model. **b** Immunofluorescence for GFP and T1α revealed mGFP signals were

not detected in T1α⁺ AECI in neither low nor high dose of TAM models. Boxed area was enlarged on the right. One representative example out of three embryos is shown. Scale bar 50 μm

directly or indirectly, during lung development, are actively pursued in our laboratory. This study and our continuing efforts will provide insight into the mechanism coordinating the TGF-β super family in regulating

organogenesis and into the understanding of the molecular mechanisms of congenital lung anomalies in human, and would provide new strategies for new therapeutic developments.

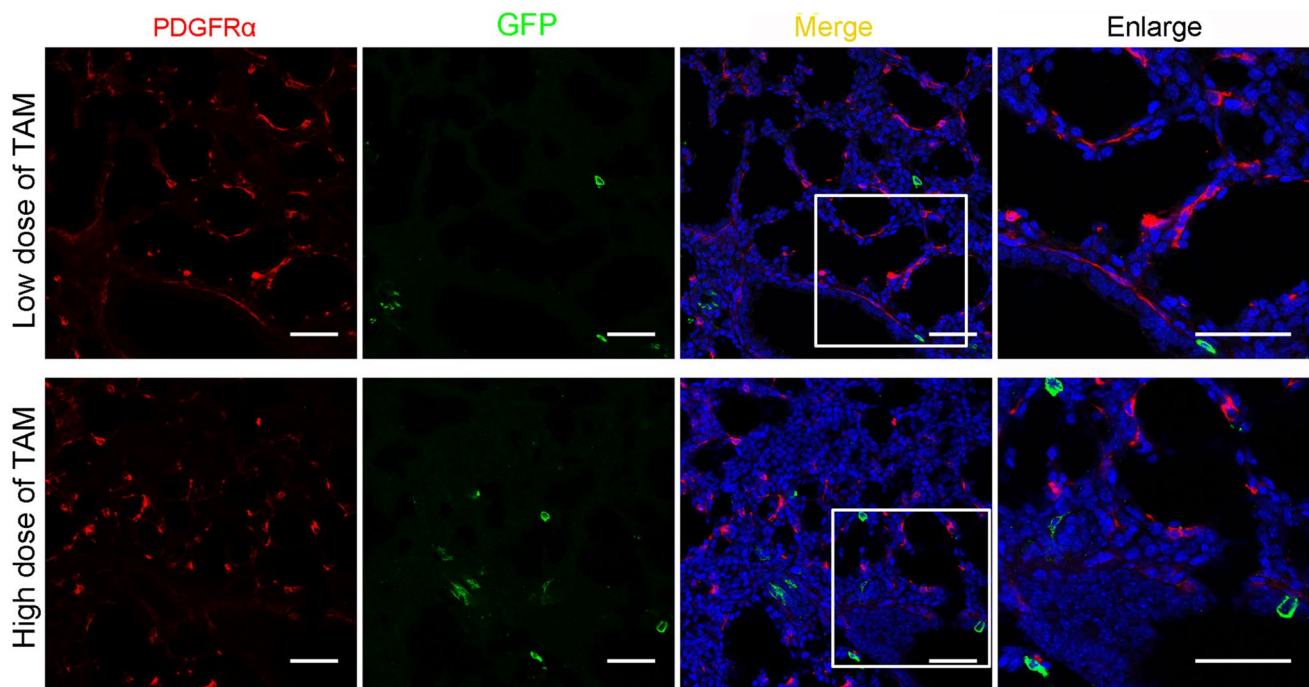


Fig. 6 *Fstl1* was not expressed in $\text{PDGFR}\alpha^+$ myofibroblasts. Immunofluorescence for $\text{PDGFR}\alpha$ and mGFP showed that mGFP signals were not detected in $\text{PDGFR}\alpha^+$ alveolar myofibroblasts in neither low

nor high dose of TAM models. Boxed area was enlarged on the right. One representative example out of three embryos is shown. Scale bar 50 μm

Materials and methods

Ethics statement

All mice were housed and cared for in a specific-pathogen free (SPF) facility at Nankai University. All experimental procedures were approved by the Institutional Animal Care of Experimental Animal Center, Nankai University. Pregnant mice were sacrificed by cervical dislocation, the embryos were isolated quickly after uterus was removed. After decapitation of the embryos, the specimens were rapidly harvested and used for subsequent experiments. All efforts were made to minimize animal suffering and all approaches were used to minimize the number of animal used.

Mouse strains

Fstl1-CreERT2 knock in mice were generated in Nanjing Biomedical Research Institute of Nanjing University. *Rosa-mT-mG* (B6.129(Cg)-Gt(ROSA)26Sortm4 (ACTB-tdTomato-EGFP)LuoJ) mice were purchased from Nanjing Biomedical Research Institute of Nanjing University (stock number: J007676). *Fstl1-CreERT2;Rosa26-mT-mG* mouse line were obtained by crossing *Fstl1-CreERT2* mice with lineage reporter *Rosa26-mT-mG* mice. The genotypes of the mice were determined by polymerase chain reaction (PCR). Sequences of the specific genotyping primer sets are as

follows: *Fstl1-CreERT2*, forward, 5'-TGCC ACGACCAAG TGACAGCAATG-3', reverse, 5'-ACTGCCCATTGCCCA AACAC-3', *Rosa26-mT-mG*, forward, 5'-CTCTGCTGCCTC CTGGCTTCT-3', reverse, 5'-CGAGGCGGATCACAAGCA ATA-3' and 5'-TCAATGGGCGGGGGTTCGTT-3'.

TAM administration

A TAM stock solution of 10 mg/ml was produced by dissolving TAM powder (T-5648, Sigma-Aldrich) in corn oil (C8267, Sigma-Aldrich). To examine recombination in embryonic lung, pregnant mice received one or several intraperitoneal injections of TAM and were sacrificed at indicated time for sectioning and analysis.

Immunofluorescence and confocal microscopy

Mouse lungs were isolated and dissected in ice cold PBS and then fixed in 10% neutral formalin for paraffin sections or in 4% paraformaldehyde for frozen sections.

Immunofluorescence was performed on 5 μm thick paraffin wax-embedded sections or frozen sections as described previously (Geng et al. 2011). For FSTL1 staining, frozen sections were treated with 3% H_2O_2 to block endogenous peroxidase and then 5% normal goat serum to block non-specific protein followed by primary antibody incubation overnight at 4 $^\circ\text{C}$. Sections were then washed and incubated

with biotinylated secondary antibodies (BA-9400; Vector Labs) followed by incubation in ABC reagent (PK-7100, Vector Labs). Antigen localization was detected with TSA fluorescein system (SAT70100KT, PerkinElmer). Primary antibodies used were anti-GFP (abcam, ab13970), α -SMA (Santa Cruz, sc-32251), CCSP (Seven Hills Bioreagents, wrab-3950), CD31 (ZSGB-BIO Origene, ZM-0568), E-cadherin (BD Biosciences, 610181), β -tub IV (BioGenex, MU178-UC), Foxj1 (eBioscience, 14-9965-82), CGRP (Millipore, AB5920), Fstl1 (R&D, MAB17381), pro-SPC (Abcam, ab170699), P63 (ZSGB-BIO Origene, ZM-0406), Endomucin (eBioscience, 14-5851), PDGF β (eBioscience, 14-1402), T1 α (Developmental Studies Hybridoma Bank, 8.1.1), NG2 (Millipore, AB5320), Mucin 5AC (Neomarkers, MS-145-P0), and PDGFR α (eBioscience, 14-1401). The Alexa Fluor 488, 555, and 633 secondary antibodies were purchased from Invitrogen. Tissue sections were viewed on a Leica SP8X TCS confocal imaging microscope (Leica Microsystems Inc).

Cell sorting and specific single cell qRT-PCR

Lungs isolated from E18.5 *Fstl1-CreERT2; Rosa26-mT-mG* embryos were minced and incubated in digestion buffer (8 mg/ml collagenase IV and 2 mg/ml Dispase II in PBS) at 37 °C for 30 min. We used epithelial cell adhesion molecular (EpCAM, CD326 PE-Cyanine 7 eBioscience 25-5791) and mGFP as the selection marker. The EpCAM⁺ mGFP⁻ and EpCAM⁺ mGFP⁺ groups were collected by fluorescence-activated cell sorting (FACS). Single cell qRT-PCR was performed by Single Cell Sequence Specific Amplification Kit (Vazyme, P621-01) according to manufacturer's instructions. Briefly, RT-PreAmp Master Mix were mixed as described by the instruction of the kit. Each cell was load into reaction tube, which concatenated 5 μ l RT-PreAmp Master Mix. The mix was then kept at -80 °C for 2 min and the reaction was carried out following the protocol: reverse transcription at 50 °C for 1 min, denaturation at 95 °C for 1 min followed by 20 consecutive cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 15 min. After the reaction, the cDNA was diluted and used for qPCR. qPCR was performed by using SYBR GreenER qPCR SuperMix Universal (Invitrogen) according to the manufacturer's protocols. Gene expression was measured relative to the endogenous reference gene, β -actin. Sequences of the specific primer sets are as follows: *β -actin*: forward, 5'-AGG CCAACCGTGAAAAGATG-3', reverse, 5'-AGAGCATAG CCCTCGTAGATGG-3', *Fstl1*, forward, 5'-TTATGATGG GCACTGCAAAGAA-3', reverse 5'-ACTGCCTTTAGA GAACCAGCC-3', *Sftpc*, forward, 5'-GAAGATGGCTCC AGAGAGCATC-3', reverse, 5'-GGACTCGGAACCAGT ATCATGC-3', *Scgbl1*, forward, 5'-CTCCTTCGCCCA

GGTTGTTATAG-3', reverse, 5'-CTCTTGTGGGAGGGT ATCC-3'.

Statistical analysis

Data were expressed as the mean \pm SEM. SPSS was used for statistical analysis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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