# Conditional knockout of ITGB4 in bronchial epithelial cells directs bronchopulmonary dysplasia

**Yu Chen**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Wang Jiang**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Jin-Mei Wang**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Xiao-Di Ma**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Di Wu**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Le-Xin Liu**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Ming Ji**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Xiang-Ping Qu**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Chi Liu**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Hui-Jun Liu**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Xiao-Qun Qin**
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
**Yang Xiang** ( xiangyang@csu.edu.cn )
Central South University Xiangya Medical College: Central South University Xiangya School of Medicine
https://orcid.org/0000-0002-7865-2565

**Research Article**

**Keywords:** ITGB4, BPD, FAK, GSK3β, SOX2

**Posted Date:** April 25th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1565262/v1

# Conditional knockout of ITGB4 in bronchial epithelial cells directs bronchopulmonary dysplasia

**Yu Chen[1],[2] Wang Jiang[1]，Jin-Mei Wang[1]，Xiao-Di Ma[1]，Di Wu[1]，Le-Xin Liu[1]，Ming Ji[1]，Xiang-Ping Qu[1], Chi Liu[1], Hui-Jun Liu[1], Xiao-Qun Qin[1], Yang Xiang[1]***

1 School of Basic Medicine, Central South University, Changsha 410078, Hunan, PR China
2 Department of Medical Laboratory, School of Medicine, Hunan Normal University, Changsha, 410013, China.
*Correspondence to: Yang Xiang, Department of Physiology, Xiangya School of Medicine, Central South University, Changsha 410008, Hunan, PR China. Tel: 86-731-82355051. Fax: 86-731-82355056. E-mail: xiangyang@csu.edu.cn.

**Abstract**

**Background:** Neonatal respiratory system disease is closely associated with embryonic lung development. Our group found that Integrin β4(ITGB4) is downregulated in the airway epithelium of asthma patients. Asthma is the most common chronic respiratory illness in childhood. Therefore, we suspect whether the deletion of ITGB4 would affect fetal lung development? In this study, we characterized the role of ITGB4 deficiency in Bronchopulmonary dysplasia (BPD).

**Methods:** ITGB4 was conditionally knocked out in CCSP-rtTA; Tet-O-Cre; $ITGB4^{f/f}$ triple transgenic mice administrating doxycycline from E7.5 to P42. Lung tissues at different developmental stages were collected for experimental detection and transcriptome sequencing. The expression of lung-development-relevant factors were evaluated by transcriptome sequencing, WB and Immunohistochemical. Bronchoalveolar lavage fluid were collected to perform Total cell and differential inflammatory cell counts. The effects of ITGB4 deficiency on lung branching morphogenesis were observed by fetal mouse lung explants culture.

**Results:** We found that $β4^{CCSP.Cre}$ mice exhibit significant lighter body weight comparing to age-matched littermate control $β4^{f/f}$, and the difference became more distinct as their age increase. ITGB4 deficiency shows no significant impact on the ratio of lung weight to body weight and the overall survival rates of mice. Deleting ITGB4 from the airway epithelial cells result in enlarged alveolar airspaces, inhibition of branching, the overlapping of epithelium cells and the impairment of cilial growth during lung development. Scanning electron microscopy showed that the airway epithelial cilia of $β4^{CCSP.Cre}$ group appear to be sparse, shortened, and lodging. Lung-development-relevant factors such as SPTPC、SOX2 significantly decreased in both mRNA and protein level. KEGG pathway analysis indicated that multiple ontogenesis-regulating-relevant pathway converge to FAK, accordingly, ITGB4 deletion decreased phospho-FAK, phospho-GSK3β, SOX2 level, and correspondingly contrary consequence was detected after treatment with GSK3β agonist（wortmannin）. Airway branching defect of $β4^{CCSP.Cre}$ mice lung explants was also partly recovered after wortmannin treatment.

**Conclusions:** Airway epithelial-specific deletion of ITGB4 contributes to lung developmental defect, which could be achieved through the FAK/GSK3β/SOX2 signal pathway.

**Keywords**: ITGB4, BPD, FAK, GSK3β, SOX2

## Introduction

The abnormal intrauterine development of the fetal lungs is closely related to the serious diseases of the neonatal respiratory system, such as BPD. Increasing evidence shows that the long-term effects of fetal lung development impairment can last till adolescence, even adulthood. Compared with full-term newborns, newborns with BPD are susceptible to airway hyperresponsiveness and facing higher incidence of respiratory disease such as asthma and chronic obstructive pulmonary disease (COPD) in their adulthood[1, 2]. At present, the etiology and pathogenesis of BPD are still unclear. The immature lung development and lung damage caused by multiple impairment, based on genetic susceptibility, in utero and after birth was generally considered the essence of BPD.[3]

With its impaction on the morphology, polarity and direction of movement of the cells, Pulmonary matrix has decisive effect on fetal lung development process including the growth of lung bud, branching, bronchi forming and alveolarization [4-6].Integrins widely distributes in cell membrane of various organs, the combination of integrins with extracellular matrix leads to the reconstruction of the actin cytoskeleton and regulates the cell survival, differentiation and proliferation[7-9]. Besides, it participates in the bidirectional signal transduction process through direct binding or functional combination with intracellular adaptor proteins, cytoplasmic tyrosine kinases, growth factor/cytokine receptors, further affecting cell behavior[10]. Therefore, its reasonable for us to speculate that，during pulmonary development，integrin of airway epithelial cells could be the bridge of the signal transmission between cells and extracellular matrix, by which it performs important functions. Integrins are a family of heterodimeric cell surface receptors which consist of α and β subunits, it has been documented that the deficiency of integrin α3 or β1 in mice manifested as bronchial branching defects and impaired epithelial cell differentiation

and maturation [11, 12]. There is no relative research over the relationship of integrin β4 and lung development.

Our previous studies have found that the expression of integrin β4 is down-regulated in asthma patients. The sequencing of the promoter region of β4 integrins sampling from asthma patients testified that high frequency base mutation exists in the fixed site among the region, and shows corresponding relation to asthma susceptibility[13]. Foregoing results suggest that the deficiency of integrin β4 could be relevant to asthma susceptibility. Although the pathogenesis of asthma has not been elaborated, its high incidence in children implies certain connection between lung development and asthma susceptibility [14].Based on the foresaid facts, we speculated that the expression defect of integrin β4 may affect fetal lung development, and has continuous effect till adolescence or even adulthood, thereby increasing the susceptibility of chronic respiratory system diseases such as asthma.

In this study, we aim to discuss the impact of conditional knockout of integrin β4 in bronchial epithelium on lung development.

## Methods

### Mouse strains and Animal treatment

All animal experimental studies were approved by Institutional Animal Care and Use Committee of Central South University (No. 2019-S102). Referring to the documented establishment of CCSP-rtTA$^{tg/-}$/TetO-Cre$^{tg/tg}$ mice expressing Cre recombinase under the control of the clara-cell secretory protein (CCSP) promoter on a C57BL/6 background[15], ITGB4$^{fl/fl}$ mice were bred with CCSP-rtTA$^{tg/-}$/TetO-Cre$^{tg/tg}$ mice to generate the CCSP-rtTA$^{tg/-}$/TetO-Cre$^{tg/-}$/ITGB4$^{fl/fl}$ triple transgenic mice.

To specifically deletes ITGB4 from the airway epithelial cells of CCSP-rtTA $^{tg/-}$/TetO-Cre$^{tg/-}$/ITGB4$^{fl/fl}$ mice, Doxycycline (Dox; 0.125/300ml in drinking water) was ingested from embryonic day 7.5 (E7.5) till postnatal day 42(P42). ITGB4$^{fl/fl}$ littermates lacking either CCSP-rt TA, TetO-Cre or both were used as controls (β4$^{f/f}$) which were given identical dosage of doxycycline.

**Cell cultures and treatment**

Incubated Human bronchial epithelial (HBE) cells, purchased from Lifeline Cell Technology (Frederick, MD, USA), in a humidified environment, under the condition of 37°C with 95% air and 5% carbon dioxide, and changed the medium every 24h, collection was performed when cells grown to 95%-100% confluence. Cells were seeded in a 6-well plate at a density of 2~5×$10^5$ cells/well and grown to 50-70% confluence, subsequently, transfected with siRNA for 48h, then treated each group with 2.5μM GSK3β agonist (wortmannin)[16] (S2758, Selleck.cn), or equal concentrations of DMSO(Sigma-Aldrich) considered as control.

**Immunostaining analysis**

Lung lobes were fixed in 4% Paraformaldehyde and embedded with paraffin. The expression of ITGB4 and SOX2 were detected by both immunofluorescent (IF) and immunohistochemical staining (IHC). Expression of other relative factors was detected by immunohistochemical staining. Antibodies used: CCSP (sc365992,Santa Cruz), ITGB4(ab182120,Abcam)，TTF1(ab76013, Abcam), SOX2(GTX101507, GeneTex), SOX9(ab185966, Abcam), FAK(40913,SAB), p-FAK(13327, SAB), GSK3β(BF8003, Affinity), phospho-GSK3β(AF2016, Affinity), sftpB(ABS21, Merck Millipore), sftpC(ab211326, Abcam).

**Western blotting**

Total protein extraction was performed using the mixture containing RIPA and protease inhibitor (100:1), and quantified by the BCA kit. Centrifuged the lysates at the condition of 12,000 rpm for 20min, 4°C, and took the supernatant for detection. Separated by 10% SDS-PAGE gels, the supernatant was then transferred to a PVDF membranes, blocked with 5% non–fat milk for 2 hours at room temperature on a shaking table.

Then, ITGB4 primary antibody (dilution 1:1000; ab182120, Abcam), SOX2(dilution 1:6000, GTX101507, GeneTex), FAK (dilution 1:1000, 40913, SAB), phospho-FAK (dilution 1:1000, 13327, SAB), GSK3β (dilution 1:1000, BF8003, Affinity), phospho-GSK3β (dilution 1:1000, AF2016, Affinity), and β-actin (dilution 1:5000; ab, Abcam) were added and incubated at 4°C overnight. The following day,

washed the membrane in TBST for 3 times, 5 mins each. Subsequently, incubated in the secondary antibody (1:5000 dilution) at room temperature for 30 min. quantified the relative band intensity using the Image Lab Analyzer software (Bio-Rad, Hercules, CA).

**Bronchoalveolar lavage**

Bronchoalveolar lavage fluid (BALF) was collected by the lavaging of the left lungs for 3 times with 0.5mL of PBS each. Lavage fluid was centrifuged at 2000 rpm at 4°C, then the total cell counts was performed. Differential blood counts: A smear of the cell pellet of the BALF was prepared, and then examined by Wright-Giemsa staining (Sigma, Germany).

**ITGB4 siRNA synthesis and transfection**

The design and synthesis of ITGB4 siRNA and nonsense siRNA were executed by Guangzhou RiboBio (RiboBio Inc., Guangzhou, China). Transfections were performed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

ITGB4 siRNA: 50-CAGAAGAUGUGGAUGAGUU-30

nonsense siRNA: 50-UUCUCCGAACGUGUCACGU-30

**Transcriptome sequencing**

Lung tissues were collected at E13.5, P2, P7, and P28 for transcriptome sequencing（PRJNA781075）. Samples divided into two groups: $\beta4^{ccsp.cre}$ mice, and the littermate $\beta4^{f/f}$ mice (considered as control). Library preparation and clustering for transcriptome sequencing of all the samples were performed by the Novogene company (Novogene, China).

**Fetal mice lung explant culture**

The E12.5 mouse lung tissue were isolated and placed onto 24-mm clear polyester membrane supports (Transwell, 0.4μm pore size; Corning, Corning, NY) to create an air-liquid interface, then cultured overnight at 37°C to allow its adherence to the filter. Culture medium (DMEM) was added only to the basal compartment [17, 18] PBS/DOX were added to $\beta4^{f/f}$ group, and PBS/DOX/DOX+wortmannin were added to $\beta4^{ccsp.cre}$ group. After been cultured for 48h, images taken by Motic

BA410EF-UPR microscope were used for new generated saccular airway branches counting, as documented[17].

**Morphological analysis**

Hematoxylin-eosin staining (H&E) was performed for assessment of Lung morphometry, using a 40× objective for six sections per mouse, with a minimum of 6 mice in each group. Using Image Pro Plus software. The mean linear intercept （MLI）and mean septal wall thickness were measured after staining with H&E [19]. Scanning electron microscope (SEM) was used for morphology observation of ciliated cells, Mice airways were fixed with glutaraldehyde (2.5%) solution for scanning electron microscopy detection at the College of Life Sciences, Hunan Normal University, Changsha, China.

**Macrophage clearance assay**

Macrophage clearance assay was conducted with clodronate Clodronate (dichloromethylene diphosphonic acid; Sigma-Aldrich) and sterile PBS-containing liposomes (vehicle) as previously described[20, 21]. $\beta4^{f/f}$ and $\beta4^{CCSP.Cre}$ mice were treated with intranasal clodronate or PBS-containing liposome vehicle every 5 days beginning at P5 until P14 or P28 for harvest. Different doses of intranasal clodronate were given respectively at P5, P10, P15, P20 and P25: 12μl, 15μl, 18μl, 21μl and 24μl. PBS-containing liposome vehicle control were used for duplicate experiments.

**Statistics**

All experiments were independently repeated for three times. Data are shown as means±SD, and were analyzed by SPSS19.0 statistical analysis (IBM, Chicago, IL), visualized by Graph Pad Prism software (Version 7.0, San Diego, CA). Differences between of two groups were determined by two-tailed unpaired Student's t-test, while that of multiple groups were tested using analysis of variance (ANOVA) or two-way ANOVA. For both statistical analyses, considering the P value $< 0.05$ as statistically significant.

**Results**

**Establishment of ITGB4 conditionally knockout mice**

To investigate the role of ITGB4 in lung development, CCSP-rtTA; Tet-O-Cre;

ITGB4$^{fl/fl}$ triple transgenic mice were generated as mentioned in method, Cre-meditated targeting excision was triggered by doxycycline, through administration orally or intraperitoneally. Doxycycline is a semisynthetic tetracycline, commonly used for gene conditional knockout. However, high dosage of Dox could be toxic for organs[22-24], while the knockout efficiency might be affected applying a low dosage, thus its necessary for us to determine the suitable dosage which efficiently knocks out ITGB4 with no negative effect on lung development.Concentration gradient was built for the screening, the effect of different concentrations of doxycycline on lung morphology and knockout efficiency was evaluated respectively by HE and IHC staining[13, 25].

At the concentration of 0.25g/300ml, ITGB4 was successfully knocked out, but the overlapping of airway epithelial cells was observed in both β4$^{CCSP.Cre}$and β4$^{f/f}$ mice, such phenomenon was eliminated after treatment with Dox of 0.0625g/300ml, but ITGB4 was not completely knocked out. Graphical analysis of staining suggested 0.125g/300ml as the final concentration using which we carried out our subsequent experiment. (Fig.1A) Further verification of ITGB4 knockout in airway epithelial cells was accomplished by WB and triple immunofluorescence staining. ITGB4 expressed in near-linear basilar stained airway cells throughout the conducting airways of β4$^{f/f}$ mice, as regards β4$^{CCSP.Cre}$ mice, ITGB4 expression was deleted in the conducting bronchi and proximal bronchioles (Fig.1A,1D). Western blot analysis of primary CCSP$^{+}$airway epithelial cells also shows that ITGB4 expression was eliminated after Dox treatment. (Fig.1B,1C)

**Airway epithelial-specific deletion of ITGB4 contribute to lung developmental defects**

**ITGB4 deficiency resulted in abnormal lung structure without affecting overall survival rate.**

We observed physiological and morphological features of mice at different development stages. β4$^{CCSP.Cre}$ mice grew poorly with significantly lower weight than age-matched littermate control β4$^{f/f}$, and the difference increased as time

prolonged. (Fig.2A,2C). But the overall survival rates and ratio of lung weight to body weight (LW/BW) were not significantly impacted by ITGB4 deficiency (Fig.2B,2D).

Lungs harvested at various developmental stages were analyzed by H&E, I F, and SEM. Although no structural difference was observed at E13.5 between $\beta4^{CCSP.Cre}$ and $\beta4^{f/f}$ mice, visible defects in branching morphogenesis emerged at E18. Transverse or coronal lung sections from $\beta4^{CCSP.Cre}$ embryos showed fewer but more dilated airway tubules (Figure.3E).Fetal lung explants culture was ca rried out to analyze the effects of ITGB4 on branching morphogenesis. The ad dition of DOX did not affect branching morphogenesis. Compared with control s, significantly less new branching occurred in $\beta4^{CCSP.Cre}$ lung explants after cul tured for 48h (Fig.9E-9L). Airway branches counts of $\beta4^{CCSP.Cre}$ lung explants was significantly less than $\beta4^{f/f}$ group after been cultured for 48h.

Histological examination of lung parenchyma harvested at various time-poi nts from P10 to P42 showed that the lung structures of $\beta4^{CCSP.Cre}$ mice manifes ted typical characteristics of alveolar simplification, as indicated by enlarged al veoli with decreased secondary septa, irregular alveolar shape with an emphyse ma-like enlargement of distal airspaces (Fig.3A,3B) The same result was report ed in another paper of our group [26]. But no obvious thickening of the alveo lar septum was found (Fig.3C). The airway epithelium of $\beta4^{CCSP.Cre}$ mice appear s to have maturation arrest and epithelial disorder, represented by overlaps of e pithelial cells. Besides, Damaged epithelium prone to denudate and slough into the airway lumen, which could be observed in both lung tissue (Fig.3D) and a lveolar lavage fluid (Fig.4A). Impairment of cilial growth or structure was obse rved using SEM, showing that the airway epithelial cilia of $\beta4^{CCSP.Cre}$ group app eared to be reduced, shortened, lodging and sparse, and large areas of non-cilia ted airway epithelium emerged (Fig.4C,4D).

**Effect of ITGB4 deficiency on development-relevant factors**

To further investigate the effect of ITGTB4 deletion on lung development, we

detected the expression of relevant factors.

TTF1 is the earliest molecule marker of lung development, regulating branching morphogenesis and the secretion of surfactant protein [27]. TTF1 protein expression was examined in multiple stages of development from E13.5 to p21 by IHC. Comparing to the control group, expression of TTF1 in $\beta4^{CCSP.Cre}$ group was markedly downregulated in the pseudoglandular stage, which is the primary branching morphogenesis stage, and in alveolar stage (Fig.5A). Besides, the expression of surfactant protein B(SftpB)(Fig.5B) and SftpC, the marker of type II alveolar epithelial cells, were also significantly decreased (Fig.5C), which suggests that conditionally knocking out ITGB4 from airway epithelium in the early stage of lung development can not only impedes alveolar epithelial cells differentiating into alveolar epithelial type II cells, but also reduced the secretion of pulmonary surfactant protein. Proximal and distal epithelial progenitor cells are labeled with SOX2 and SOX9. Both IHC and IF showed that SOX2 expression is significantly reduced inducing by ITGB4 defect (Fig.5E,5F), while the expression of SOX9 remain unchanged (Fig.5D). It shows that the specific knockout of ITGB4 in the embryonic stage has a significant effect on the development of the proximal lung, However, the unaltered expression of SOX9 suggested that SOX9 might not be involved in the impairment of the distal lung development.

**ITGB4 defect induced Bronchopulmonary dysplasia, irrelevant with inflam mation.**

Lung inflammation is also an important factor in bronchopulmonary dysplasia [28].To investigate whether inflammation involved in the development of bronchopulmonary dysplasia, we carried out differential blood counts in BALF, and macrophage clearance assay on $\beta4^{CCSP.Cre}$ mice, in which clodronate liposomes were used to deplete macrophage, and mice treated with PBS-loaded liposomes were used as control. The results showed that basically no inflammatory cells were observed in BALF, and overlapping of airway epithelial cells and alveolar cavity enlargement of similar degree appeared in the lung tissues of both group treated with clodronate

liposomes or PBS-loaded liposomes (Fig.4B), indicating that the bronchopulmonary dysplasia of the ITGB4 conditional knockout mice is irrelevant with inflammation.

**Transcriptome of Airway epithelial-specific deletion of ITGB4**

To further explore the reason ITGB4 deficiency induced bronchopulmonary dysplasia, transcriptome sequencing was performed on four different developmental stages: Pseudoglandular stage (E13.5), Saccular stage (P2), Alveolar stage I(P7), Alveolar stage II(P28).[5, 29, 30]

Differential expressed genes (DEGs) between the $\beta4^{f/f}$ and $\beta4^{CCSP.Cre}$ groups were screened out by high-throughput transcriptome sequencing in Each paired group of all 4 stages (Fig.6A), Combining GO and KEGG pathway enrichment analysis, DEGs were mainly enriched in extracellular matrix、Collagen trimer、contractile fiber function at the Pseudoglandular stage. At the Alveolar stage I, DEGs were mainly enriched in cilium, microtubule bundle formation. As for Alveolar stage II, DEGs were mainly enriched in Collagen fibril organization、growth factor activity and cellular response to growth factor stimulus(Fig.6B,6C). KEGG enrichment analyses suggested that most of DEGs were involved in focal adhesion、cell adhesion molecules、regulation of actin cytoskeleton、 small cell lung cancer and pathways in cancer(Fig.6D).

Visualizing the transcription results to line graphs containing 4 time points of lung development, We analyzed the expression of ITGB4 and relevant proteins including: cell markers: airway epithelial cells—Foxj1[31], type I alveolar epithelial cells—Aquaporin-5 [32]、vascular endothelial cells—PECAM-1[33] and SftpC, SOX2, SOX9 [27]. Together with Growth factors related to lung development: Transforming Growth Factor Beta-1 (TGFβ1)[34], Epidermal Growth Factor (EGF) [27], Sonic hedgehog (SHH) [35], and SftpB [36], Laminin 5 (LN-5) [37, 38]—The ligand of ITGB4. First of all, ITGB4 showed a decline at all time points, indicating that the integrin β4 conditional knockout mouse model we constructed was successful (Fig.7A). and Foxj1, SftpC, SOX2, SftpB (Fig.7B, 7C, 7D, 7E) of $\beta4^{CCSP.Cre}$ mice were significantly decreased after b

irth, While the expression of Aquaporin-5, PECAM-1, SOX9, TGFβ1, EGF, SHH, and Laminin 5 (Fig.7F,7G,7H,7I,7J,7K,7L) did not change significantly.

Aforementioned changes of mRNA expression were consistent with protein. (Fig.5) functionally, it also proved the results of the previous GO and KEGG enrichment analysis.

**ITGB4 might regulate Lung development through FAK/GSK3β /SOX2 signal pathway**

Mapping the DEGs to the KEGG pathway analysis (Figure 6B), it can be seen that the focal adhesion kinase (FAK) is located at the intersection of multiple signal transmission pathways, involving cell migration, adhesion and other physiological activities, and is important for meditation of signaling downstream of integrins and growth factor receptors[39].Combining the KEGG pathway analysis with transcriptome sequencing, we found that glycogen synthase kinase 3β(GSK3β) and downregulated SOX2 is also the downstream molecule of FAK, thus, we speculated that FAK/GSK3β/SOX2 could be the underlying mechanism of bronchopulmonary dysplasia induced by integrin β4 conditional knockout.

The results indicated no significant changes in the expression of FAK and GSK3β upon loss of β4 expression. But the decreased expression of phospho-FAK, phospho-GSK3β and SOX2 is obviously detected (Fig. 8A, 8B,8C). The experiments performed on HBE transfected with β4 siRNA or nonsense siRNA agreed with foresaid results (Fig. 9A ,9B,). Correspondingly, protein levels of phospho-FAK、phospho-GSK3β and SOX2 recovered after treated with wortmannin(Fig.9C ,9D,). Branching morphogenesis was evaluated by cultures of fetal lung explants treated with or without wortmannin (Fig.9E-9O). Although wortmannin treatment did not fully rescued airway branching defects (Fig.9M,9N), partial recovering was achieved compared with control group without wortmannin treatment. (Fig.9K,9L). Taken together, these data indicate that deficiency of β4 integrin disrupts lung development through FAK/GSK3β /SOX2 signal pathway.

**Discussion**

Epithelial-mesenchymal interactions have an important role in developing and in adult lungs[40]. Integrins are transmembrane proteins and surface adhesion receptors，it mediated the interaction of cells and the extracellular matrix, Which is important for the formation, maintenance, and repair of tissues as well as for other biological processes such as the metastasis of cancer cells.Integrin β4 has unique structure and subcellular localization, making it the most unusual molecule of all the β integrin subunits, the cytoplasmic domain of which is bigger than other β subunits, including a juxtamembrane domain and two pairs of type III Fn-like repeats [41-43] Deleting integrin β4 in airway epithelial cells with 0.125g/300ml DOX at E7.5 results in abnormal lung development and weight loss, but the overall survival rate was not affected, which indicates that ITGB4 defect will only cause growth retardation, but not death. It has been published that ITGB4 participate in mammary gland development and maintain the integrity of mammary architecture, ITGB4 disruption impedes branching morphogenesis in immortalized normal breast cell.[44]

In our research, A variety of ITGB4 knockout-induced lung development defects were observed at multiple time-points, including altered branching morphogenesis, impaired alveolarization, differentiation defects of epithelial cell and cilia disorders, indicating that integrin β4 expressed in airway epithelial cells has multiple functions in different stages of lung development, and its reasonable for us to speculate that these manifestation could exist during the whole lifespan of $\beta4^{CCSP.Cre}$ mice.

The morphology changes were confirmed by the downregulation of corresponding genes expression: Deleting integrin β4 in airway epithelial cells with DOX at E7.5 results in the downregulation of TTF1 in the primary branching morphogenesis stage and alveolar stage, and downregulation of SOX2 in alveolar stage, which could leads to damage to branching, and the decrease of alveolar epithelial cells number and surfactant protein secretion, as its molecule markers—sftpB, sftpC also expressed at lower level according to what we observed in the cultures of fetal lung explants (Fig.9A-9D) and staining analysis (Fig.3E,5B,5E). FOXJ1 is a transcription factor major in regulation of motile cilia growth, whose

downregulation provided evidence for cilia damage (Fig.4C,4D). Integrin-β4 may be essential in regulating the effects of injury and repair on EMT in airway epithelial cells via influencing both the cell adhesion to ECM and cells' physical phenotypes through RhoA signaling pathway.[45] As the cell surface adhesion receptors, integrin β4 absence in the airway epithelium weakened the intercellular connection, led to the airway epithelial detachment, via which resulted in the impairment of airway epithelial integrity, explaining the mass of detached airway epithelial cells collected in BALF of $\beta4^{CCSP.Cre}$ mice.

It has been reported that ITGB4 defect enhanced pulmonary inflammation in LPS-induced ALI mice,[46] as for our research, we barely observed inflammatory cells in the BALF of $\beta4^{CCSP.Cre}$ mice, while macrophage clearance assay also proved that no spontaneous inflammation aroused after ITGB4 depletion, and LW/BW was not statistically different from that of the control group, these clues indicated that the manifestations of ITGB4 epithelium-specific knockout has no relevance with inflammation.

On the base of what we had found out, we could assure that ITGB4 defect could affect lung development in various ways, in order to Clarify the underlying mechanisms, transcriptome sequencing of lung samples from different development stages was carried out. Consistent with molecule changes previous described, GO and KEGG pathway analysis shows that DEGs mainly enriched in the cilium and adhesion-related pathways. Signaling networks visualized from KEGG pathway analysis shows that FAK is positioned at the upstream cross-spot of the multiple pathways through which ITGB4 performing its function. The cytoplasmic domain of ITGB4 includes various of tyrosine phosphorylation site, A large number of cytoskeletal and signaling proteins have been reported binding to those sites positioned at the tail of cytoplasmic domain. ITGB4 affects cell signaling through modulating cell signaling pathways involving transmembrane protein kinases like FAK and receptor tyrosine kinases.[47, 48]. The maintenance of proper cell motility calls for dynamic regulation of adhesion signaling. FAK contributes to cytoskeletal reorganization, adhesion, migration, and survival[49]. SOX2 is of great significance

for the differentiation of proximal progenitors into various progeny,[50] whose defects results in the loss of the mature secretory and ciliated lineages of lung airways.[51, 52] Its impact could sustains till adulthood.Consistently, we observed that the expression of SOX2 was downregulated after ITGB4 knockout. SOX2 is also located at the downstream of FAK signaling pathway, thus, we inferred that the impairments of lung development induced by ITGB4 knockout could be realized through FAK/GSK3β/SOX2 pathway. Despite the fact that no significant changes of FAK and its downstream molecule, GS3Kβ, were observed, the expression of phospho-FAK and phospho-GSK3β were downregulated in β4-silenced HBE(Fig.9A,9B) and lung tissues (Fig.8A,8B,8C) sampled from β4-defect mice compared to each control group. FAK plays an important role in integrin-mediated cell migration，Reduced FAK phosphorylation effectively inhibited FAK mediated cell migration.[53] our group found that migration and proliferation of ITGB4 deficiency cells were noticeably inhibited, along decreased cytoskeleton stabilization .[26] Therefore, we hypothesized that the increased alveolar space, branch inhibition and epithelial cell overlap of β4-defect mice may be caused by the decreased expression of P-FAK resulting in the inhibition of cell migration. Treatment of wortmannin restored the expression of phosphor-GSK3β and SOX2(Fig.9C,9D), in addition, although branching defect still existed, it was partially rescued (Fig.9M,9N,9O), suggesting that GSk3β executes its function by regulating the expression of downstream molecules.The ITGB4/FAK/SOX2/HIF-1α signal pathway was confirmed by researchers studying the regulation of ECM1 on GC cell metastasis and glucose metabolism in gastric cancer.[54]

It has been reported that deleting β1 integrin in lung epithelium beginning at E10.5 results in abnormal lung development with a 100% fatality rate at the age of 16 weeks.[12] Based on our observation, Knockout of ITGB4 in airway epithelial cells had no effect on overall survival rate, besides, in the previous research of our group, we observed that after LPS-treatment, the mRNA expression of anti-inflammatory factors, including IL-10 and ARGA-1, were significantly reduced in the lung tissue of $\beta4^{CCSP.Cre}$ mice comparing to wildtype.[46] Under the stimulation of HDM, it's more

likely for $\beta4^{CCSP.Cre}$ mice to acquire airway hyperresponsiveness and asthma than wildtype[43]. These results suggest the different role of β4 than β1 in lung development. Though it's not lethal, during lung developmental stages, ITGB4 defects might affects multiple signaling pathways and the resistance of fetal lung to the environment changes inside and outside the uterus, leading to pulmonary dysplasia and be associated with many other pulmonary dysplasia-related diseases. Furthermore, it has long term effect on pulmonary function, making it more susceptible to acquired chronic respiratory system diseases.

In our research, we attribute the defects of lung development to the impacts ITGB4 has on FAK/GSK3β/SOX2 pathway, however, ITGB4 itself is a key factor of epithelial-mesenchymal interactions, the execution of its function could involve multiple signaling pathway, thus its impact might not only be restricted to lung development, it could also be related to other pulmonary disease. Therefore, research on fetal lung development mechanisms can not only pave the way for the exploration of therapeutic targets of neonatal lung diseases, but also have important guiding significance for the prevention and treatment of adult congenital or acquired lung diseases.

.

## Declarations

**Ethics approval and consent to participate**

The study has been granted the IRB of Third Xiangya Hospital，Central South University approval.(2019-S102)

**Consent for publication**

the authors listed have approved the manuscript that is enclosed.

**Availability of data and material**

All data generated or analysed during this study are included in this published article

**Competing interests**

The authors declare that they have no competing interests

**Funding**

This work was supported by National Natural Science Foundation of China （Grant ID 81670002）

**Authors' contributions**

YC：designed the study, performed the research, analysed data, and wrote the paper.
WJ： collected the data，analysed data
J-MW，DW，L-XL：collected the data
X-DM： technical editing of the manuscript
MJ，X-PQ, CL, H-JL，X-QQ, YX：discussed the results，critically reviewed the study proposal

**Acknowledgement**

We gratefully acknowledge Dr. Arnoud Sonnenberg and Dr. Jeffrey A. Whitsett for their kindly providing the useful transgene mice.

**Abbreviations**

ITGB4: Integrin β4; BPD: bronchopulmonary dysplasia; COPD: chronic obstructive pulmonary disease; HBE: human bronchial epithelial; SHH: Sonic hedgehog;IF:immunofluorescent ; IHC: immunohistochemical staining; H&E:Hematoxylin-eosin staining; BALF: Bronchoalveolar lavage fluid ; SftpB: surfactant protein B; SftpC: surfactant protein C; FAK: focal adhesion kinase; GSK3β:glycogen synthase kinase 3β; Foxj1: airway epithelial cells; Aquaporin-5: type I alveolar epithelial cells; PECAM-1: vascular endothelial cells; TGFβ1: Transforming Growth Factor Beta-1; EGF: Epidermal Growth Factor; SHH: Sonic hedgehog; LN-5:Laminin 5

## reference


1. Madurga A, Mizikova I, Ruiz-Camp J, Morty RE. Recent advances in late lung development and the pathogenesis of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol. 2013; 305: L893-905.
2. Pasha AB, Chen XQ, Zhou GP. Bronchopulmonary dysplasia: Pathogenesis and treatment. Exp Ther Med. 2018; 16:4315-4321.
3. Gien J, Kinsella JP. Pathogenesis and treatment of bronchopulmonary dysplasia. Curr Opin Pediatr. 2011; 23: 305-13.
4. Geng Y, Dong Y, Yu M, Zhang L, Yan X, Sun J, et al. Follistatin-like 1 (Fstl1) is a bone morphogenetic protein (BMP) 4 signaling antagonist in controlling mouse lung development. Proc Natl Acad Sci U S A. 2011; 108: 7058-63.
5. Herriges M, Morrisey EE. Lung development: orchestrating the generation and regeneration of a complex organ. Development. 2014; 141: 502-13.
6. Pozzi A, Zent R. Extracellular matrix receptors in branched organs. Curr Opin Cell Biol. 2011; 23: 547-53.
7. Brown CW, Amante JJ, Goel HL, Mercurio AM. The alpha6beta4 integrin promotes resistance to ferroptosis. J Cell Biol. 2017; 216: 4287-97.
8. D G Woodside SL, M H Ginsberg. Integrin Activation. Thromb Haemost. 2001 Jul; 86(1):316-23.:
.
9. Yoshikazu Takada XY, Scott Simon. the integrins. Genome Biology. 2007; 8:215.2-215.8.
10. Lefrancais E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. Nature. 2017; 544: 105-9.
11. Nicolaou N, Margadant C, Kevelam SH, Lilien MR, Oosterveld MJ, Kreft M, et al. Gain of glycosylation in integrin alpha3 causes lung disease and nephrotic syndrome. J Clin Invest. 2012; 122: 4375-87.
12. Plosa EJ, Young LR, Gulleman PM, Polosukhin VV, Zaynagetdinov R, Benjamin JT, et al. Epithelial beta1 integrin is required for lung branching morphogenesis and alveolarization. Development. 2014; 141: 4751-62.
13. Xiang Y, Zhou XY, Tan YR, Tan ML, Liu HJ, Liu C, et al. Analysis on the relevance of asthma susceptibility with the alteration of integrin beta 4 expression. PLoS One. 2014; 9: e95533.
14. Sun Ying GZ, Shuyan Gu, Jisheng Zhao. How Much Do We Know about Atopic Asthma: Where Are We Now? Cellular & Molecular Immunology. 2006; 3(5):321-32.
15. Ceteci F, Ceteci S, Zanucco E, Thakur C, Becker M, El-Nikhely N, et al. E-cadherin controls

bronchiolar progenitor cells and onset of preneoplastic lesions in mice. Neoplasia (New York, NY). 2012; 14: 1164-77.

16. Wang L, Li N, Shi FX, Xu WQ, Cao Y, Lei Y, et al. Upregulation of AMPK Ameliorates Alzheimer's Disease-Like Tau Pathology and Memory Impairment. Mol Neurobiol. 2020; 57: 3349-61.

17. Blackwell TS, Hipps AN, Yamamoto Y, Han W, Barham WJ, Ostrowski MC, et al. NF-kappaB signaling in fetal lung macrophages disrupts airway morphogenesis. J Immunol. 2011; 187: 2740-7.

18. Prince LS, Okoh VO, Moninger TO, Matalon S. Lipopolysaccharide increases alveolar type II cell number in fetal mouse lungs through Toll-like receptor 4 and NF-kappaB. Am J Physiol Lung Cell Mol Physiol. 2004; 287: L999-1006.

19. Chao CM, Yahya F, Moiseenko A, Tiozzo C, Shrestha A, Ahmadvand N, et al. Fgf10 deficiency is causative for lethality in a mouse model of bronchopulmonary dysplasia. J Pathol. 2017; 241: 91-103.

20. Sinha M, Sen CK, Singh K, Das A, Ghatak S, Rhea B, et al. Direct conversion of injury-site myeloid cells to fibroblast-like cells of granulation tissue. Nat Commun. 2018; 9: 936.

21. Zaynagetdinov R, Sherrill TP, Polosukhin VV, Han W, Ausborn JA, McLoed AG, et al. A critical role for macrophages in promotion of urethane-induced lung carcinogenesis. J Immunol. 2011; 187: 5703-11.

22. D'Souza A, Flynn K, Chhabra S, Dhakal B, Hamadani M, Jacobsen K, et al. Rationale and design of DUAL study: Doxycycline to Upgrade response in light chain (AL) amyloidosis (DUAL): A phase 2 pilot study of a two-pronged approach of prolonged doxycycline with plasma cell-directed therapy in the treatment of AL amyloidosis. Contemp Clin Trials Commun. 2017; 8: 33-8.

23. Gauthier F, Bertrand JR, Vasseur JJ, Dupouy C, Debart F. Conjugation of Doxorubicin to siRNA Through Disulfide-based Self-immolative Linkers. Molecules. 2020; 25(11):2714.

24. Kim JE, Park AY, Lee SY, Park YL, Whang KU, Kim HJ. Comparison of the Efficacy of Azithromycin Versus Doxycycline in Acne Vulgaris: A Meta-Analysis of Randomized Controlled Trials. Ann Dermatol. 2018; 30: 417-26.

25. Ustiyan V, Wert SE, Ikegami M, Wang IC, Kalin TV, Whitsett JA, et al. Foxm1 transcription factor is critical for proliferation and differentiation of Clara cells during development of conducting airways. Dev Biol. 2012; 370: 198-212.

26. Chi Y, Chen Y, Jiang W, Huang W, Ouyang M, Liu L, et al. Deficiency of Integrin β4 Results in Increased Lung Tissue Stiffness and Responds to Substrate Stiffness via Modulating RhoA Activity. Frontiers in Cell and Developmental Biology. 2022; 10.

27. Volckaert T, De Langhe SP. Wnt and FGF mediated epithelial-mesenchymal crosstalk during lung development. Dev Dyn. 2015; 244: 342-66.

28. Jung E, Lee BS. Late-Onset Sepsis as a Risk Factor for Bronchopulmonary Dysplasia in Extremely Low Birth Weight Infants: A Nationwide Cohort Study. Sci Rep. 2019; 9: 15448.

29. Mullassery D, Smith NP. Lung development. Semin Pediatr Surg. 2015; 24:152-155.

30. Schittny JC. Development of the lung. Cell Tissue Res. 2017; 367:427-444.

31. Lukas Didon RKZ, Ion Wa Chao, Matthew S Walters, Rui Wang, Neil R Hackett , Ronald G Crystal. RFX3 Modulation of FOXJ1 regulation of cilia genes in the human airway epithelium. Respiratory Research. 2013; 14(1): 70.

32. Tonghui Ma NF, Yuanlin Song, Michael A. Matthay, A.S. Verkman. Lung fluid transport in aquaporin-5 knockout mice. The Journal of Clinical Investigation. 2000 Jan 1; 105(1): 93–100.

33. H. M. DeLisser MC-S, R. M. Strieter, M. D. Burdick, C. S. Robinson, R. S. Wexler, J. S. Kerr, C. Garlanda, J. R. Merwin, J. A. Madri, S. M. Albelda. Involvement of Endothelial PECAM-1/ CD31 in Angiogenesis.

Am J Pathol. 1997; 151(3): 671–677.
34. Dabovic B, Chen Y, Choi J, Davis EC, Sakai LY, Todorovic V, et al. Control of lung development by latent TGF-beta binding proteins. J Cell Physiol. 2011; 226: 1499-509.
35. Herriges JC, Verheyden JM, Zhang Z, Sui P, Zhang Y, Anderson MJ, et al. FGF-Regulated ETV Transcription Factors Control FGF-SHH Feedback Loop in Lung Branching. Dev Cell. 2015; 35: 322-32.
36. Bi G, Wu L, Huang P, Islam S, Heruth DP, Zhang LQ, et al. Up-regulation of SFTPB expression and attenuation of acute lung injury by pulmonary epithelial cell-specific NAMPT knockdown. FASEB J. 2018; 32: 3583-96.
37. Ogawa T, Tsubota Y, Hashimoto J, Kariya Y, Miyazaki K. The short arm of laminin gamma2 chain of laminin-5 (laminin-332) binds syndecan-1 and regulates cellular adhesion and migration by suppressing phosphorylation of integrin beta4 chain. Mol Biol Cell. 2007; 18: 1621-33.
38. Zahir N, Lakins JN, Russell A, Ming W, Chatterjee C, Rozenberg GI, et al. Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. J Cell Biol. 2003; 163: 1397-407.
39. Hitchcock IS, Fox NE, Prevost N, Sear K, Shattil SJ, Kaushansky K. Roles of focal adhesion kinase (FAK) in megakaryopoiesis and platelet function: studies using a megakaryocyte lineage specific FAK knockout. Blood. 2008; 111: 596-604.
40. Badri L, Walker NM, Ohtsuka T, Wang Z, Delmar M, Flint A, et al. Epithelial interactions and local engraftment of lung-resident mesenchymal stem cells. Am J Respir Cell Mol Biol. 2011; 45: 809-16.
41. Geuijen CA, Sonnenberg A. Dynamics of the alpha6beta4 integrin in keratinocytes. Mol Biol Cell. 2002; 13: 3845-58.
42. Litjens SH, Koster J, Kuikman I, van Wilpe S, de Pereda JM, Sonnenberg A. Specificity of binding of the plectin actin-binding domain to beta4 integrin. Mol Biol Cell. 2003; 14: 4039-50.
43. Liu C, Yuan L, Zou Y, Yang M, Chen Y, Qu X, et al. ITGB4 is essential for containing HDM-induced airway inflammation and airway hyperresponsiveness. J Leukoc Biol. 2018; 103: 897-908.
44. S Stahl SW, J C Jones. the role of laminin-5 and its receptors in mammary epithelial cell branching. Journal of Cell Science. 1997 Jan; 110 ( Pt 1):55-63.
45. Mei-Ling Tan W-JH, Yue Wang, . Integrin-β4 regulates the dynamic changes of phenotypic characteristics in association with epithelial-mesenchymal transition (EMT) and RhoA activity in airway epithelial cells during injury and repair. International Journal of Biological Sciences. 2022; 18(3): 1254-1270: 1254-70

46. Jiang W, Wang JM, Luo JH, Chen Y, Pi J, Ma XD, et al. Airway epithelial integrin beta4-deficiency exacerbates lipopolysaccharide-induced acute lung injury. J Cell Physiol. 2021.
47. Bugaj LJ, Spelke DP, Mesuda CK, Varedi M, Kane RS, Schaffer DV. Regulation of endogenous transmembrane receptors through optogenetic Cry2 clustering. Nat Commun. 2015; 6: 6898.
48. Khurana S, Schouteden S, Manesia JK, Santamaria-Martinez A, Huelsken J, Lacy-Hulbert A, et al. Outside-in integrin signalling regulates haematopoietic stem cell function via Periostin-Itgav axis. Nat Commun. 2016; 7: 13500.
49. MICHAEL D.SCHALLER CAB, BRADLEY S.COBB, RICHARD R.VINES,ALBERT B.REYNOLDS, J.THOMAS PARSONS. pp125FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Cell Biology. 1992; 89(11):5192-6.
50. Goissis MD, Cibelli JB. Functional characterization of SOX2 in bovine preimplantation embryos. Biol Reprod. 2014; 90(2):30.

51. Que J, Luo X, Schwartz RJ, Hogan BL. Multiple roles for Sox2 in the developing and adult mouse trachea. Development. 2009; 136: 1899-907.
52. Tompkins DH, Besnard V, Lange AW, Keiser AR, Wert SE, Bruno MD, et al. Sox2 activates cell proliferation and differentiation in the respiratory epithelium. Am J Respir Cell Mol Biol. 2011; 45: 101-10.
53. Cai GQ, Zheng A, Tang Q, White ES, Chou CF, Gladson CL, et al. Downregulation of FAK-related non-kinase mediates the migratory phenotype of human fibrotic lung fibroblasts. Exp Cell Res. 2010; 316: 1600-9.
54. Gan L, Meng J, Xu M, Liu M, Qi Y, Tan C, et al. Extracellular matrix protein 1 promotes cell metastasis and glucose metabolism by inducing integrin beta4/FAK/SOX2/HIF-1alpha signaling pathway in gastric cancer. Oncogene. 2018; 37: 744-55.

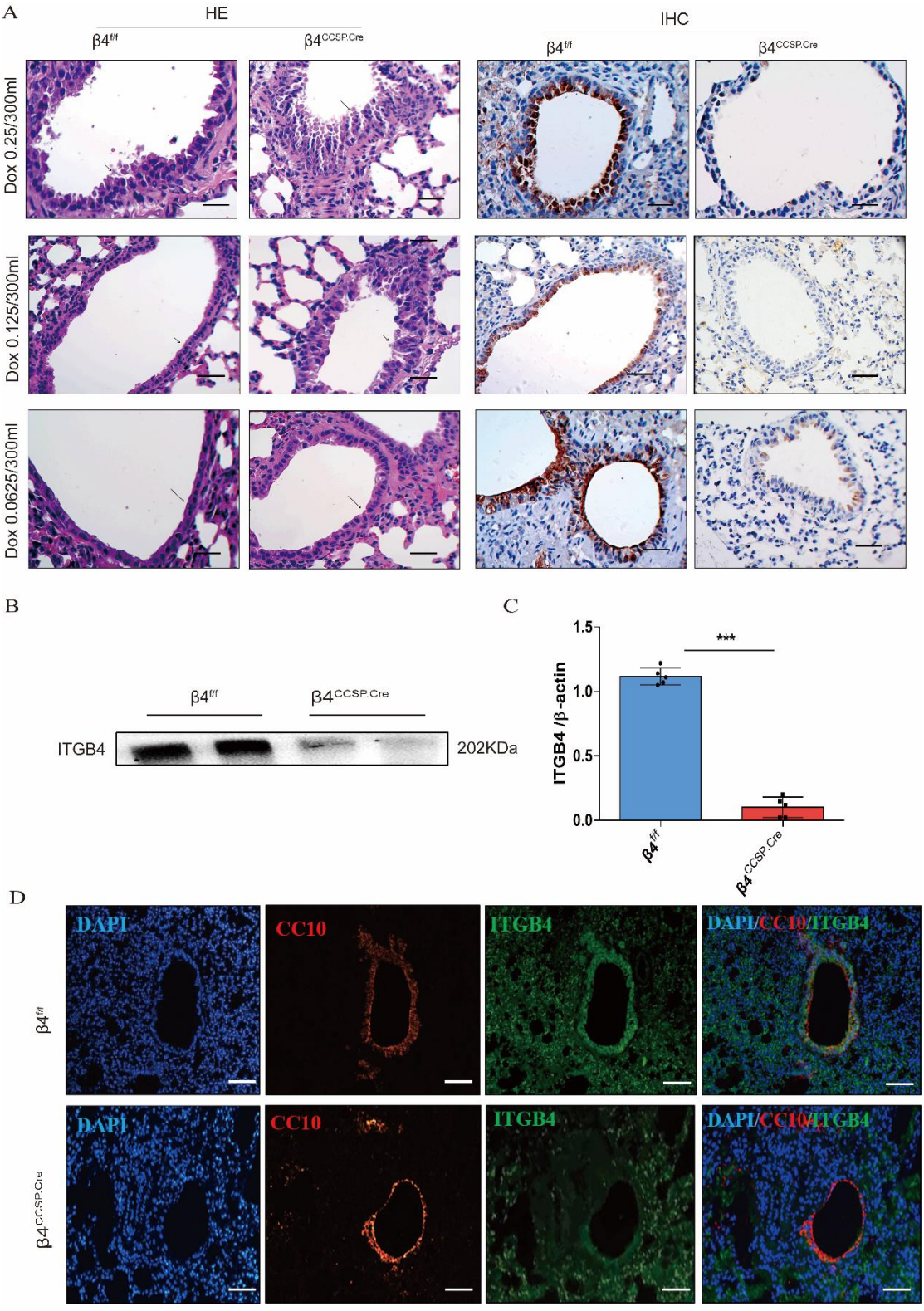


**Figure.1. Screening of Appropriate doxycycline concentration and detection of ITGB4 expression in airway epithelial cells of $\beta4^{CCSP.Cre}$ and $\beta4^{f/f}$ mice.**

（A）Doxycycline concentration was screened with immunohistochemical and H&E staining, representative airway epithelial image is shown（×400 magnifica

tion; scale bar, 25μm） (B) ITGB4 protein expression from $CCSP^{+}$ airway epithelial cells was detected by Western blot. (C)Quantification WB analysis ***P< 0.001. (D)ITGB4 deficiency validation via immunofluorescence. Co-localization of CCSP and ITGB4 was performed in lung sections. CCSP and ITGB4 were stained with red and green fluorescent separately. DAPI was used to stain cell nuclei (blue) （×200 magnification; scale bar, 50μm）

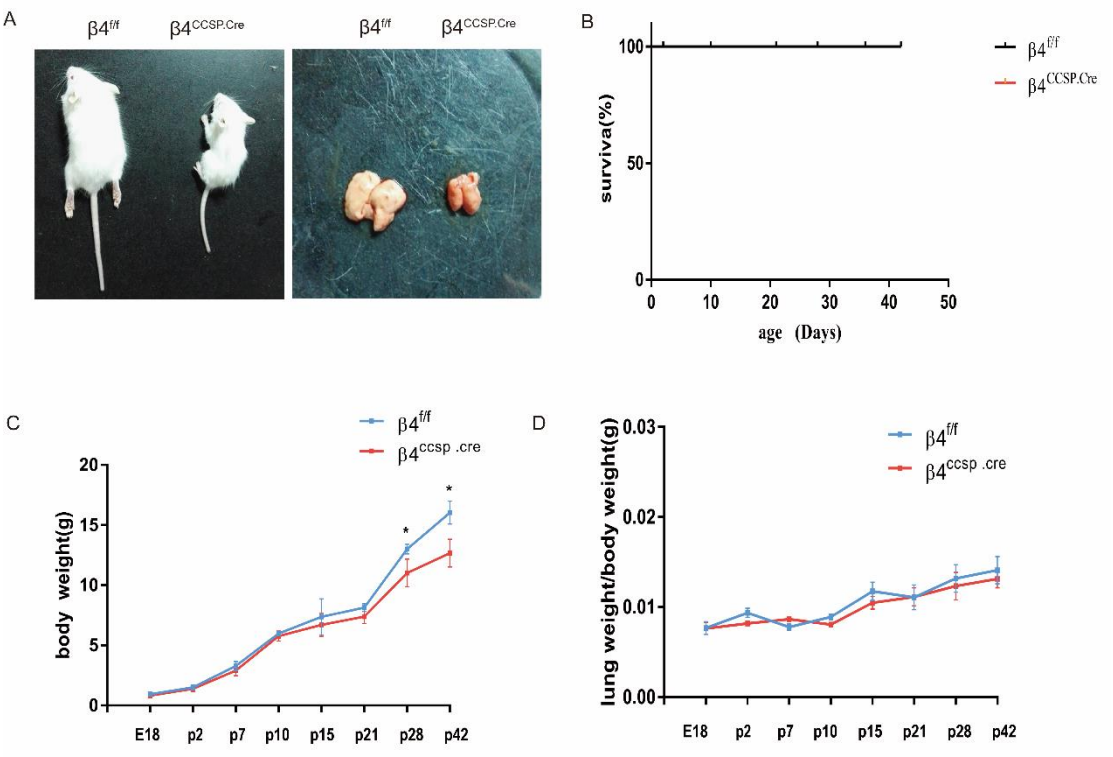


**Figure. 2. ITGB4 deficiency induced abnormal lung structure without affecting overall survival rates.** (A)Gross appearance and lungs of P21 $β4^{CCSP.Cre}$ and $β4^{f/f}$ mice (B) kaplan–Meier survival curve for $β4^{CCSP.Cre}$ and $β4^{f/f}$ mice (n=15mice/group). (C) Growth curves for $β4^{CCSP.Cre}$ and $β4^{f/f}$ mice (n=15mice/group) (D) LW/BW curves for $β4^{CCSP.Cre}$ and $β4^{f/f}$ mice (n=15mice/group)（* P<0.05）

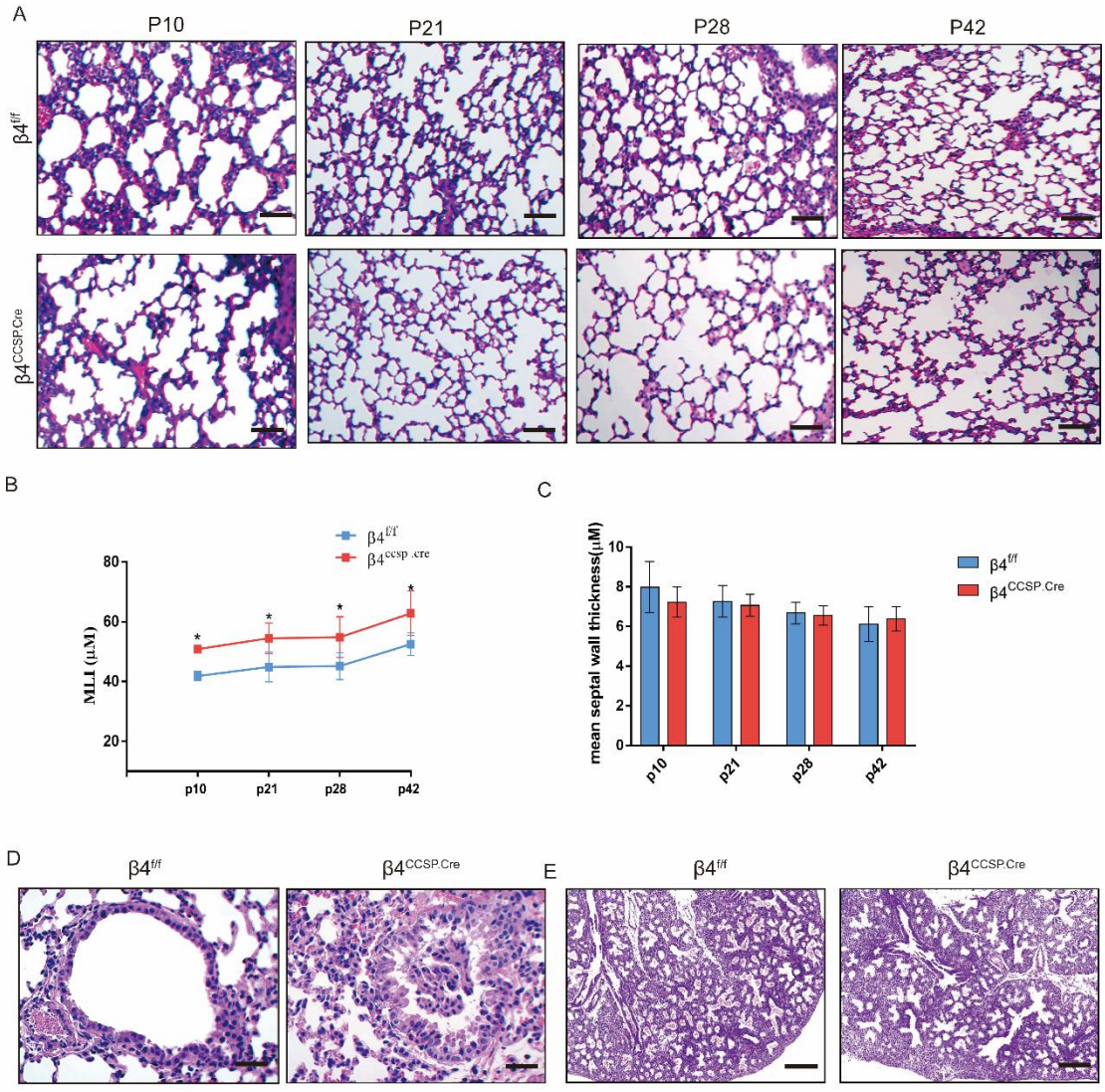


**Figure.3. ITGB4 conditional knockout induced dilated airspaces、inhibited branching and multilayer epithelium.**

(A) H&E-stained paraffin sections of P10 、P21、P28 and P42 mice lungs. (n =6 mice/group；×100 magnification; scale bar, 100μm) (B) The mean linear in tercept is greater in $\beta4^{CCSP.Cre}$ versus $\beta4^{f/f}$ mice (n=6 mice/group, 10 sections p er mouse). MLI of P10 、P21、P28 and P42 mice.（* P<0.05） (C) Quantific ation of alveolar septa at P10、P21、P28 and P42 (D). H&E-stained paraffin s ections of P28 $\beta4^{f/f}$ and $\beta4^{CCSP.Cre}$ mice for the observation of epithelium impai rments. (×400 magnification, scale bar, 25μm) (E)H&E-stained paraffin sections of $\beta4^{f/f}$ and $\beta4^{CCSP.Cre}$ lungs at E18. Decreased branching and the number of ai rspaces per $mm^2$ is present in E18 $\beta4^{CCSP.Cre}$ lungs compared with $\beta4^{f/f}$ lungs.

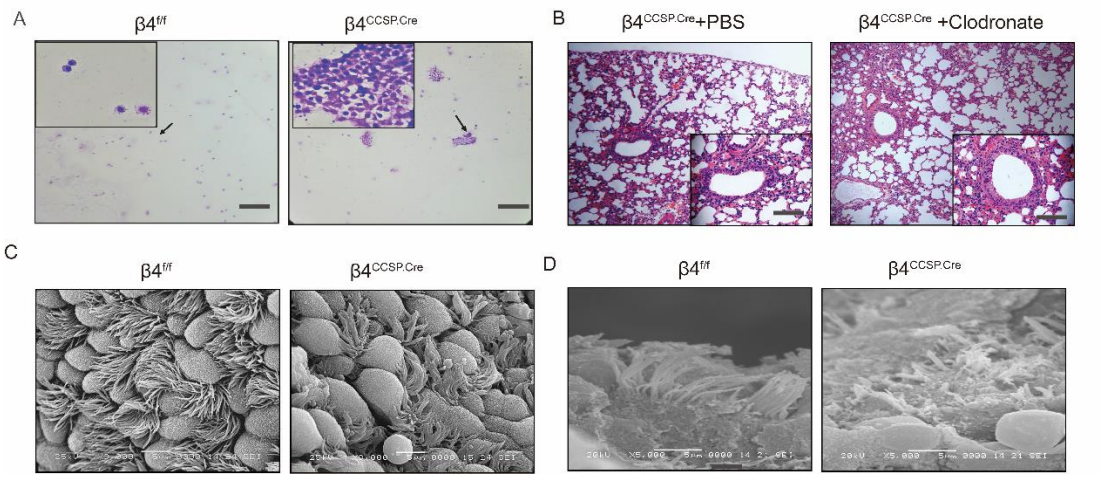


**Figure.4. Airway epithelial-specific deletion of ITGB4 contribute to epithelium and ci lia defects.** (A) denudated Airway epithelial in the balf (×100 magnification, sca le bar, 100$\mu$m) (B) multiple layers of airway epithelium and enlarged alveolar airspaces appear both in the clodronate liposomes treated group and PBS-loade d liposomes treated group. (C. D) The scanning electron microscopy (SEM) sh owing micrographs of the epithelium of the trachea in $\beta4^{CCSP.Cre}$ mice and $\beta4^{f/f}$ mice. The data represents the cumulative results of 6 independent experiments. the airway epithelial cilia of $\beta4^{CCSP.Cre}$ group appeared to be lodging(C), reduce d, shortened(D) (n=6 C: ×3000magnification, D: ×5000magnification scale bar, 5$\mu$m)

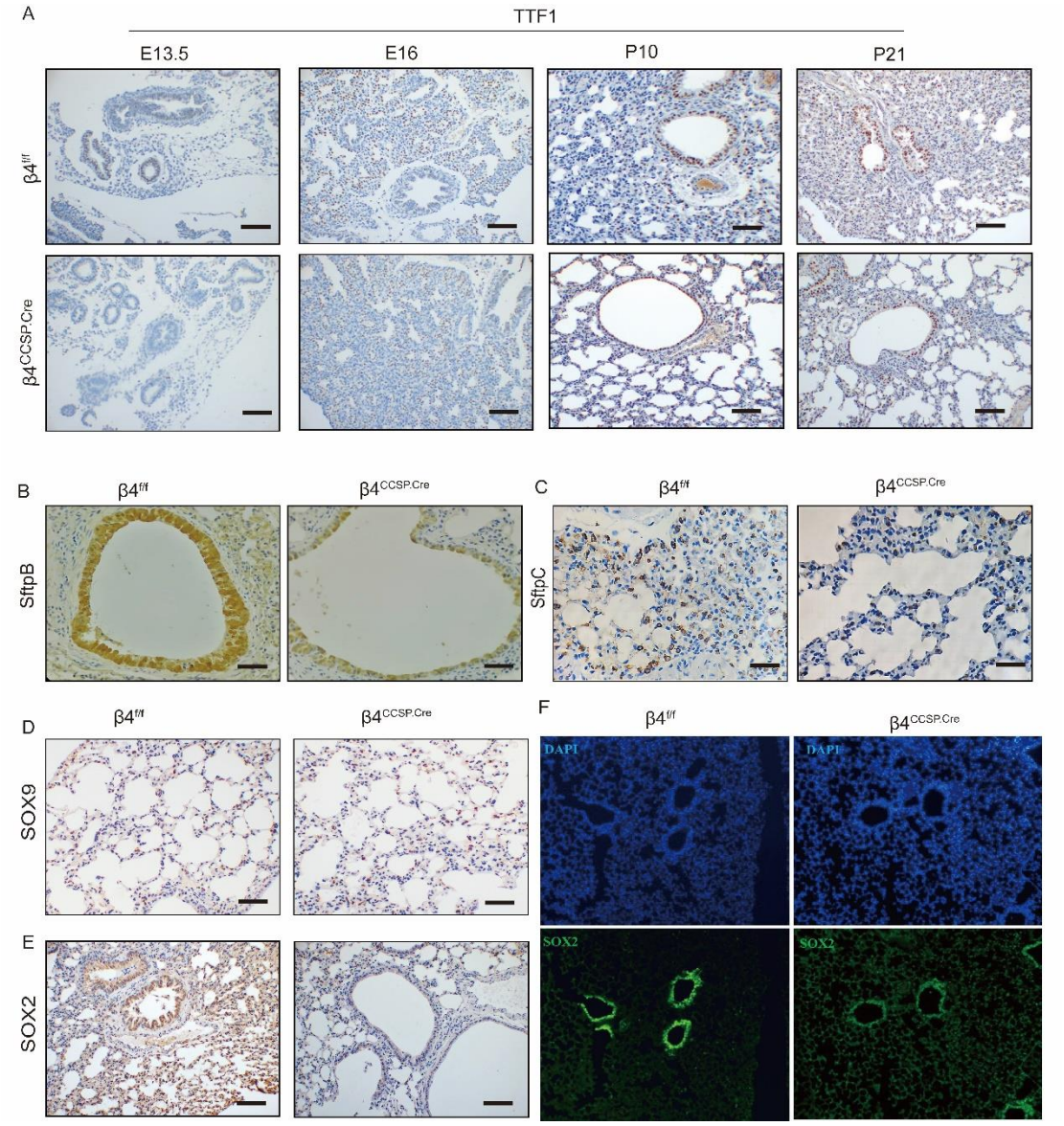


**Figure.5. Effect of ITGB4 defects in airway epithelial on development-related molecules.** (A) Immunohistochemical staining of TTF1 in the airway epithelium at E13.5、E16、P10、P21(×100 magnification, scale bar, 100$\mu$m) (B-D) Immunohistochemical staining of sftpB, sftpC and SOX9 in the airway epithelium at P42 (×400 magnification, scale bar, 25$\mu$m). (E-F) Immunohistochemical staining and Immunofluorescence detection for SOX2 (×200 magnification, scale bar, 50$\mu$m). SOX2 was stained with green fluorescent separately. DAPI was used to stain cell nuclei (blue).

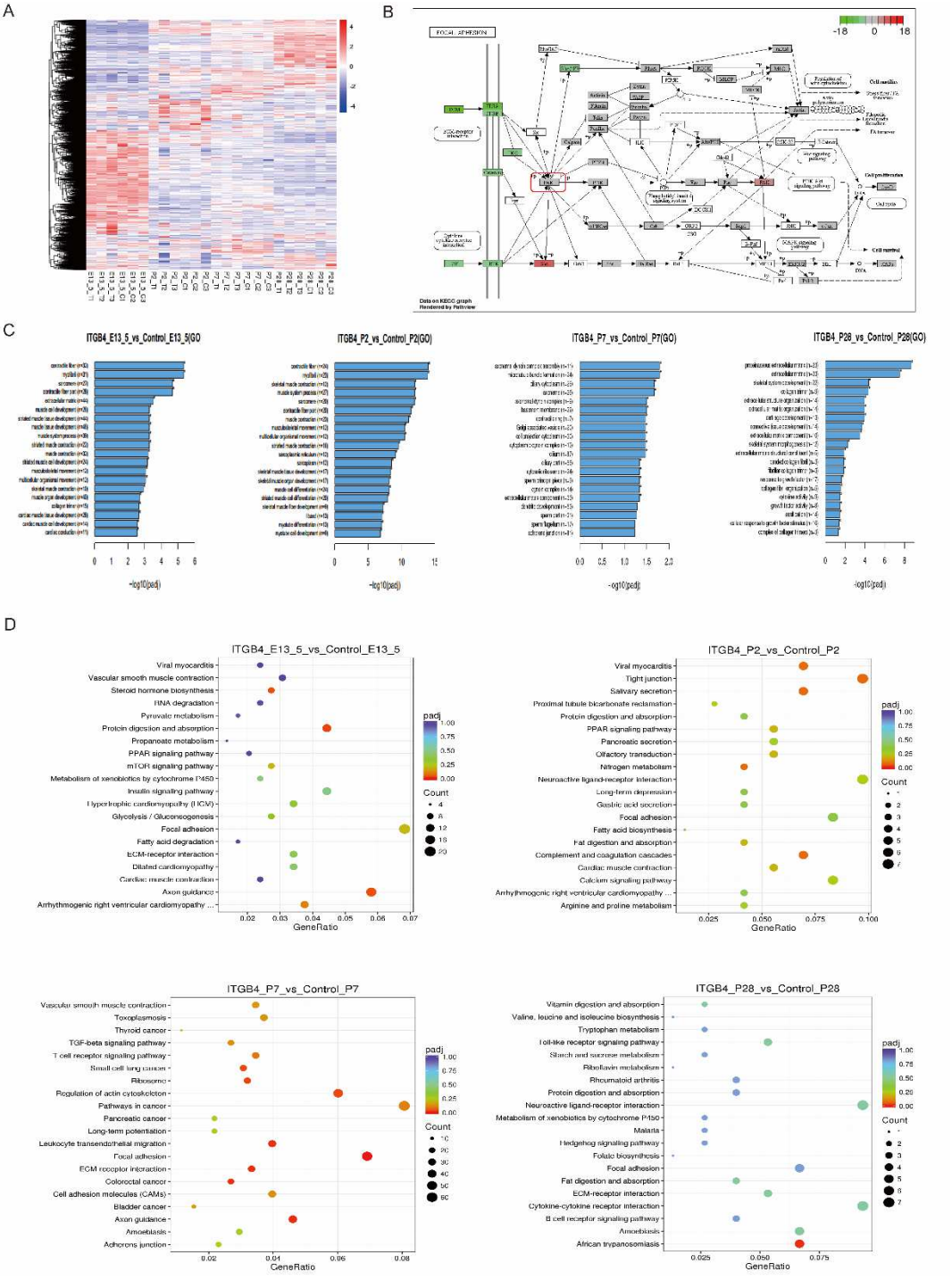


**Figure.6. Transcriptome sequencing of lung tissue from ITGB4 defects mice and wildtype mice.** (A)Cluster heatmap of differential gene expression in $\beta4^{f/f}$ and $\beta4^{CCSP.Cre}$ group at E13.5、E2、P7、P28. red = upregulation, green = downregulation. (B)Focal Adhesion map. Positive regulation is highlighted in red; negative regulation is highlighted in green. (C)GO Functional enrichment analysis in $\beta4^{f/f}$ an

d $\beta4^{CCSP.Cre}$ group at E13.5、E2、P7、P28. (D)A bubble chart shows the top 20 enriched GO terms. The size of the dot represents the proportion of genes, which is positively associated with the proportion of corresponding enrichment items. The change in color from dark blue to red represents a change in p value from high to low.

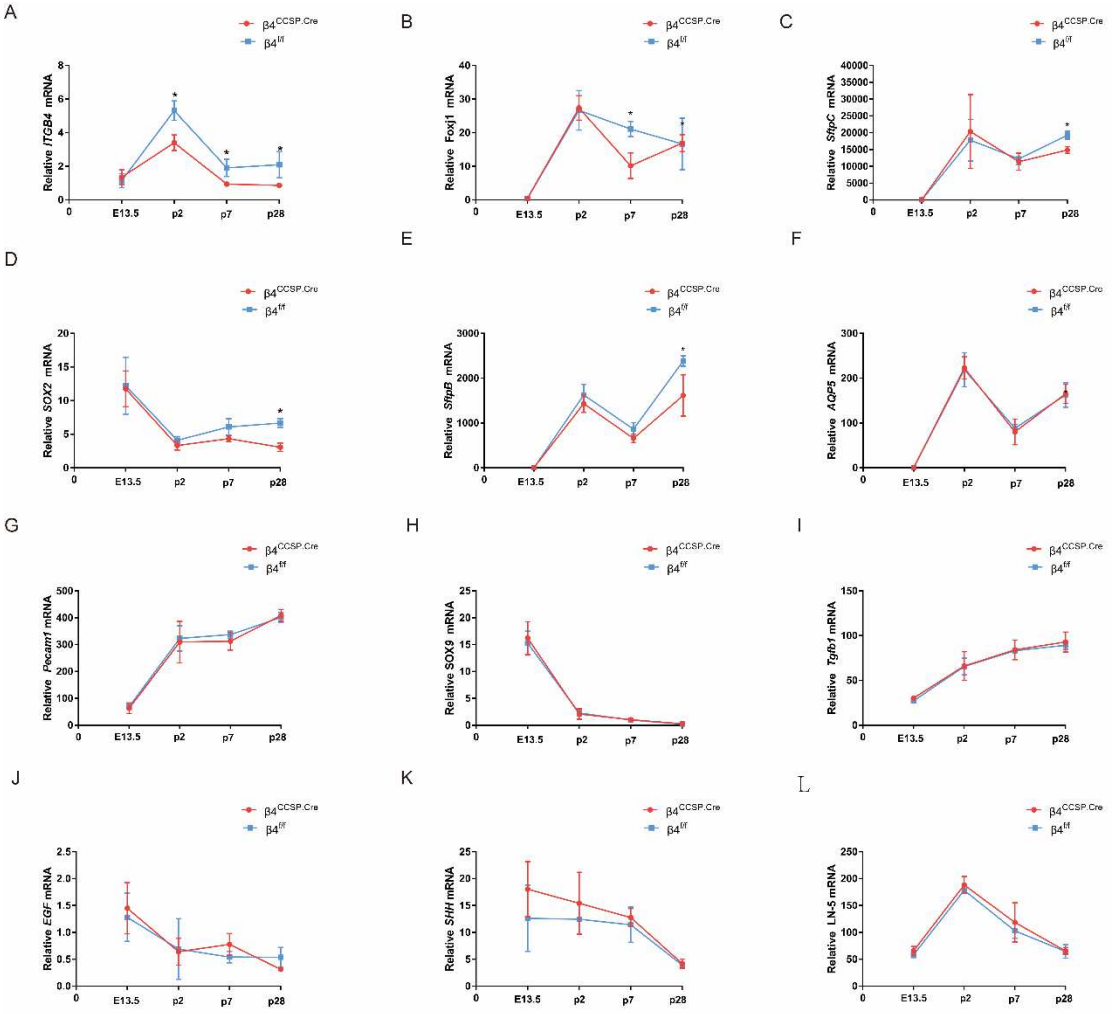


**Figure.7 The mRNA expression levels of development-associated genes at different time-points.** (A-L)The line graphs of mRNA expression levels of ITGB4、FOXJ1、AQP5、SFTPC、PECAM-1、SFTPB、EGF、TGFB1、SOX2、SOX9、LN-5、SHH at E13.5、P2、P7、P28 concluded from transcriptome sequencing.（* $P<0.05$）

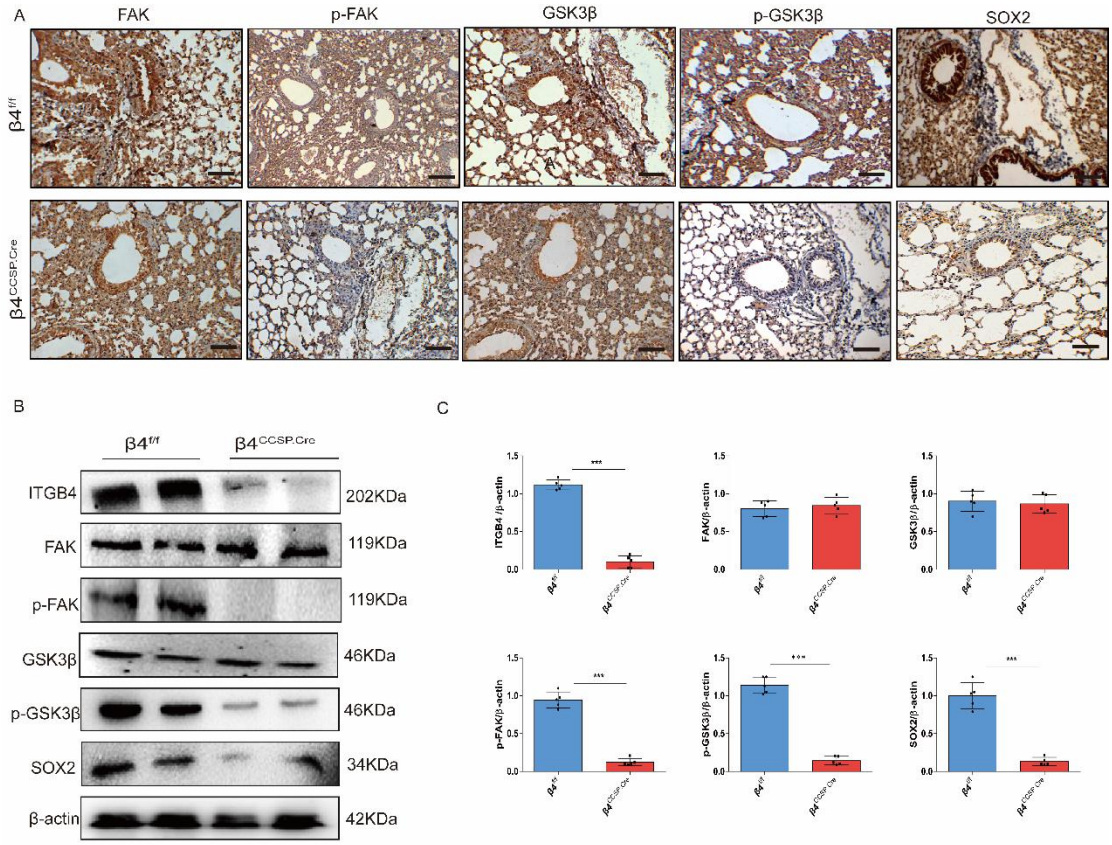


**Figure.8. The effects of ITGB4 deletion on FAK/GSK3β/SOX2 pathway in mice.**

(A) The immunohistochemical staining of FAK, phospho-FAK, GSK3β, phospho -GSK3β and SOX2 at P28 (B) Western blotting detection of the expression of ITGB4、FAk、phospho-FAK、GSK3β、phospho-GSK3β、SOX2 at P28. (C) Qu antification of western blotting. (n=6 ***P<0.001)

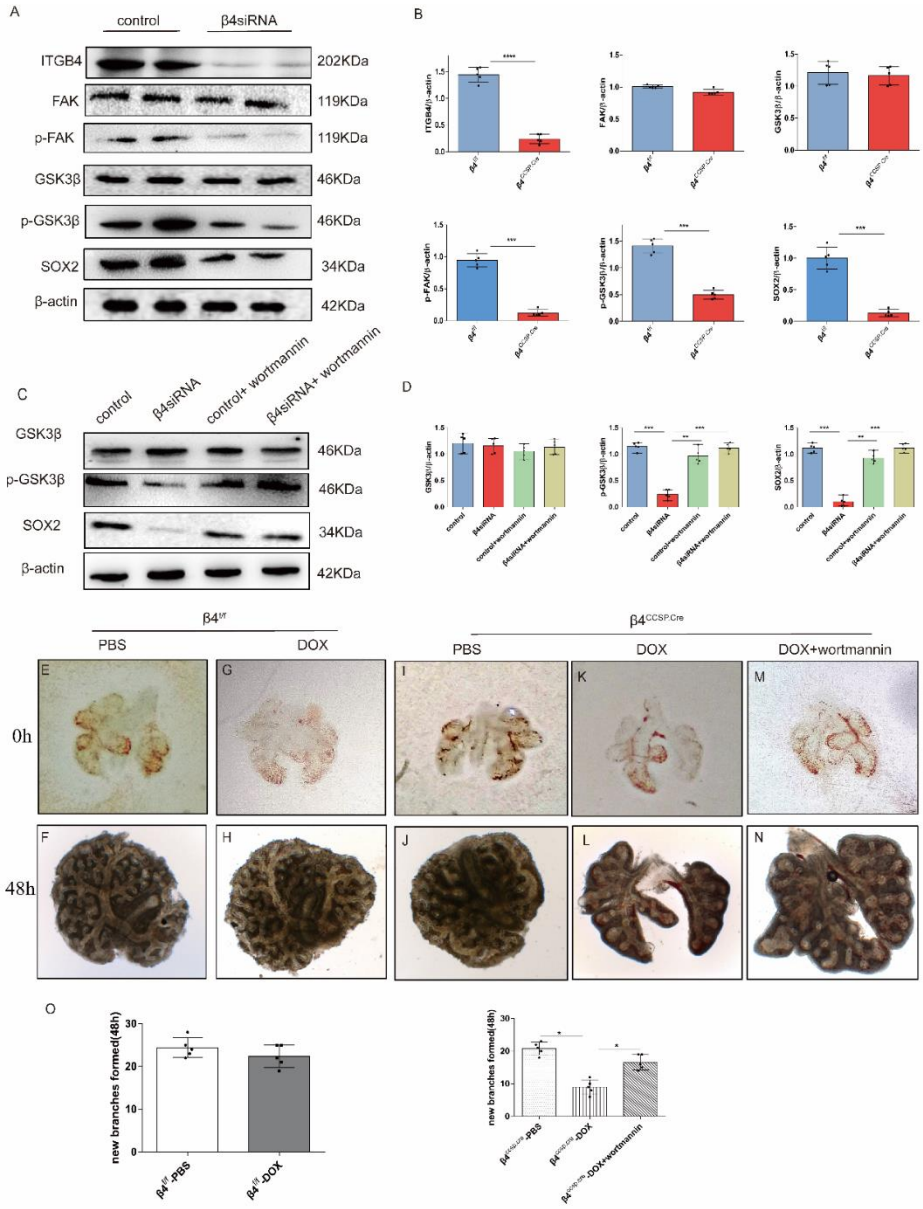


**Figure.9. ITGB4 influence Lung development through FAK/GSK3β/SOX2 si gnal pathway.** Silencing ITGB4 by small interfering RNA (siRNA) transfection in HBE cells. Experimental groups were treated with 25 μM Wortmannin. Co ntrol group were prepared with equivalent concentrations of DMSO. ITGB4、F AK、phospho-FAK、GSK3β、phospho-GSK3β、SOX2 expression was detected by (A、C)western blotting (B、D)Quantification of western blotting.(*P<0.05; * *P<0.01; ***P<0.001) (E-H) Culture of fetal lung explants from E12 $\beta4^{f/f}$ mic e, DOX was used for elimination of the effect of itself on branching morphog enesis (I-N) Culture of fetal lung explants from E12 $\beta4^{CCSP.Cre}$ mice, showing d ecreased branches comparing to control, and recovered after wortmannin treatm

ent.(O) Quantification of the new generated branches