Title
Pbx1 activates Fgf10 in the mesenchyme of developing lungs

Journal
Genesis, 52(5)

ISSN
1526-954X

Authors
Li, Wei
Lin, Chieh-Yu
Shang, Ching
et al.

Publication Date
2014-05-01

DOI
10.1002/dvg.22764

Peer reviewed

genesis 52:399–407 (2014)

LETTER

Pbx1 Activates Fgf10 in the Mesenchyme of Developing Lungs

Wei Li,[1] Chieh-Yu Lin,[1] Ching Shang,[1] Pei Han,[1] Yiqin Xiong,[1] Chien-Jung Lin,[1] Jing Yang,[1,2] Licia Selleri,[3] and Ching-Pin Chang[1,2,4,5]*

[1]Division of Cardiovascular Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, California

[2]Krannert Institute of Cardiology and Division of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana

[3]Department of Cell and Developmental Biology, Weill Medical College of Cornell University, New York

[4]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana

[5]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana

Received 7 June 2013; Revised 20 February 2014; Accepted 21 February 2014

Summary: Insufficiency of surfactants is a core factor in respiratory distress syndrome, which causes apnea and neonatal death, particularly in preterm infants. Surfactant proteins are secreted by alveolar type II cells in the lung epithelium, the differentiation of which is regulated by Fgf10 elaborated by the adjacent mesenchyme. However, the molecular regulation of mesenchymal Fgf10 during lung development has not been fully understood. Here, we show that Pbx1, a homeodomain transcription factor, is required in the lung mesenchyme for the expression of *Fgf10*. Mouse embryos lacking *Pbx1* in the lung mesenchyme show compact terminal saccules and perinatal lethality with failure of postnatal alveolar expansion. Mutant embryos had severely reduced expression of *Fgf10* and surfactant genes (*Spa*, *Spb*, *Spc*, and *Spd*) that are essential for alveolar expansion for gas exchange at birth. Molecularly, Pbx1 directly binds to the *Fgf10* promoter and cooperates with Meis and Hox proteins to transcriptionally activate *Fgf10*. Our results thus show how Pbx1 controls *Fgf10* in the developing lung. genesis 52:399–407, 2014.

Key words: Pbx1; Fgf10; mesenchyme; lung development

INTRODUCTION

Abnormalities in alveolar surfactant synthesis can cause respiratory distress syndrome (RDS), which affects ~1% of the newborns and is the leading cause of death in preterm infants (Ballard *et al*. 2003; Clements and Avery, 1998; Rodriguez *et al*., 2002). RDS usually develops in the first 24 h after the birth of premature babies with developmental insufficiency of pulmonary surfactant synthesis and structurally immature lungs (Clements and Avery, 1998; Gower and Nogee, 2011; Rodriguez *et al*., 2002; Yurdakok, 2004). Genetic abnormalities of producing surfactant proteins (Spa, Spb, Spc, and/or Spd) can result in this syndrome (Beers *et al*., 2000; Gower and Nogee, 2011; Nkadi *et al*., 2009; Nogee, 2004).

Pulmonary surfactant is a surface-active lipoprotein complex (phospholipoprotein) secreted by alveolar type II cells in the lung (Hawgood, 2004; Nogee, 2004; Whitsett and Weaver, 2002). The surfactant, comprised

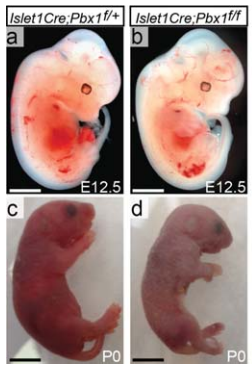
*Correspondence to: Ching-Pin Chang, M.D., Ph.D., Associate Professor of Medicine, 1800 N. Capitol Ave, E400, Indianapolis, IN 46202.
E-mail: changcp@iu.edu

Contract grant sponsor: C.P.C. was supported by the American Heart Association (AHA, Established Investigator Award), March of Dimes Foundation (MOD), the Oak Foundation, the California Institute of Regenerative Medicine (CIRM), Lucile Packard Children's Foundation, Stanford Child Health Research Institute, National Institutes of Health (R01HL085345, R01HL121197, R01HL118087), Indiana University (IU) School of MedicineIU Health Strategic Research Initiative, and the IU Physician-Scientist Initiative (endowed by Lilly Endowment, Inc.); W.L. was supported by fellowships from the Oak Foundation and the Stanford Child Health Research Institute; Y.X. by fellowships from the Oak Foundation, AHA, and Lucile Packard Children's Foundation; C.S. by MOD.

Published online 2 March 2014 in
Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/dvg.22764

of proteins and lipids with hydrophilic and hydrophobic regions, reduces the surface tension of the alveoli and thereby facilitate and maintain the expansion of the lung (Cardoso, 2001; Halliday, 2008; Mendelson, 2000). During lung development, the fibroblast growth factor Fgf7 and Fgf10 are secreted from the mesenchyme to activate the proliferation of adjacent alveolar type II cells that express the surfactant genes (*Spa*, *Spb*, *Spc*, and *Spd*; Cardoso *et al*., 1997; Chelly *et al*., 1999; Hyatt *et al*., 2004; Mason *et al*., 2002; Ramasamy *et al*., 2007), and the differentiation of lung epithelial progenitors is also regulated by Fgf10 produced by the distal mesenchyme (Volckaert *et al*., 2013).

Our studies demonstrate that the transcription factor Pbx1 functions within the lung mesenchyme to regulate Fgf10 expression. Pbx1 belongs to the family of TALE (three amino acid loop extension)-class homeodomain transcription factors that cooperate with Hox and Meis homeodomain proteins to regulate target gene expression (Chang *et al*., 1995, 1996). In embryos, Pbx1 partners with Meis/Hox proteins to facilitate the patterning of great arteries and cardiac outflow tract (OFT; Chang *et al*., 2008; Stankunas *et al*., 2008b). Here, we show that mice lacking Pbx1 in the lung mesenchyme display neonatal death with inability of lung expansion and consequent respiratory failure. Transcriptional regulation of Fgf10 by Pbx1 regulates the crosstalk between lung mesenchyme and alveolar epithelium during lung development.



e Frequency of live *Islet1Cre;Pbx1*$^{f/f}$ (From *Islet1Cre;Pbx1*$^{f/+}$ x *Pbx1*$^{f/f}$)

Age	Expected	Observed
E12.5	0.25	0.29 (N=7/24)
E13.5	0.25	0.24 (N=5/21)
E16.5	0.25	0.21 (N=7/34)
E17.5	0.25	0.26 (N=5/19)
E18.5	0.25	0.20 (N=2/10)
P0	0.25	0.00 (N=0/24)
>P0	0.25	0.00 (N=0/36)

FIG. 1. *Islet1Cre;Pbx1*$^{f/f}$ mice die after birth. (**a–d**) Gross morphology of control (*Islet1Cre;Pbx1*$^{f/+}$) and mutant (*Islet1-Cre;Pbx1*$^{f/f}$) mice at E12.5 (a,b) and P0 (c,d). Size bars for (a,b): 2 mm. Size bars for (c,d): 8 mm. (**e**) Frequency of live *Islet1Cre;Pbx1*$^{f/f}$ embryos harvested at different embryonic and postnatal dates.

RESULTS

Islet1Cre; Pbx1$^{f/f}$ Mice Die at Birth

Pbx1-null (*Pbx1*$^{-/-}$) mice display defects in the septation of aorta and pulmonary artery (Chang *et al*., 2008). To study Pbx1 function in cardiac progenitor cells for aortopulmonary septation, we deleted *Pbx1* by crossing mice carrying a loxP-flanked allele of *Pbx1* (*Pbx1*^{f}) with mice harboring *Islet1Cre*, whose Cre activity is present in cardiac progenitor cells of the secondary heart field essential for cardiac OFT development (Cai *et al*., 2003). Mutant *Islet1Cre;Pbx1*$^{f/f}$ embryos developed grossly normal up to birth without changes in weight, but the pubs died immediately after birth (Fig. 1a–e; data not shown).

The Lungs of *Islet1Cre;Pbx1*$^{f/f}$ Mice Fail to Expand at Birth

To determine the cause of lethality, we dissected the newborn pubs at P0 and found that *Islet1Cre;Pbx1*$^{f/f}$ mice failed to expand the lungs and contained no air in the lungs (Fig. 2a,b). Neither was there expansion of or air in the lungs of *Islet1Cre;Pbx1*$^{f/f}$ pups after caesarian section at E18.5 (Fig. 2c,d). The casting of airways at E17.5 showed that the airway structure was normal in *Islet1Cre;Pbx1*$^{f/f}$ embryos (Fig. 2e–h). These findings suggest that the absence of gas exchange in the lungs of mutant mice may contribute to the death of mutant pubs. We then examined the development of embryos. At E13.5, the cardiac OFT of *Islet1Cre;Pbx1*$^{f/f}$ mice was normally separated into aorta and main pulmonary artery (Fig. 2i,j). Furthermore, there were no gross abnormalities of brain, heart, kidney, liver, stomach, intestine, esophagus, trachea, and thymus (data not shown). These observations suggest a defect in lung development that may lead to neonatal lethality of the *Islet1Cre;Pbx1*$^{f/f}$ mice.

Pbx1 is Essential for Lung Development

We examined the morphology and histology of lungs of the *Islet1Cre;Pbx1*$^{f/f}$ mice. At birth (P0), the lungs of mutant mice had normal number of lung lobes (data not shown) but were grossly smaller in size (Fig. 3a,b) and didn't have any air in the alveoli (Fig. 2a,b). At E16.5, the lungs of mutant mice showed compacted terminal saccules (Fig. 3c,d). However, the mutant lungs were normal in weight (Fig. 3e).

We then asked if Pbx1 was absent in the lungs of *Islet1Cre;Pbx1*$^{f/f}$ mice and used immunostaining to directly examine Pbx1 protein distribution in the lung tissues of mutant mice. Interestingly, in the normal E16.5 lungs, Pbx1 proteins were abundant in the lung mesenchyme but absent in the epithelium (Fig. 3f). In contrast, in the lungs of E16.5 *Islet1Cre;Pbx1*$^{f/f}$ mice, Pbx1 proteins were nearly absent in the lung mesenchyme (Fig. 3g), suggesting that IsletCre directs the deletion of *Pbx1* in lung mesenchymal cells and that Pbx1 is essential in lung mesenchymal cells for alveolar expansion at birth.

Because surfactant proteins produced by alveolar type II cells are critical for forming a lipoprotein complex in the alveoli to lower surface tension and enable alveolar expansion for gas exchange (Mendelson,

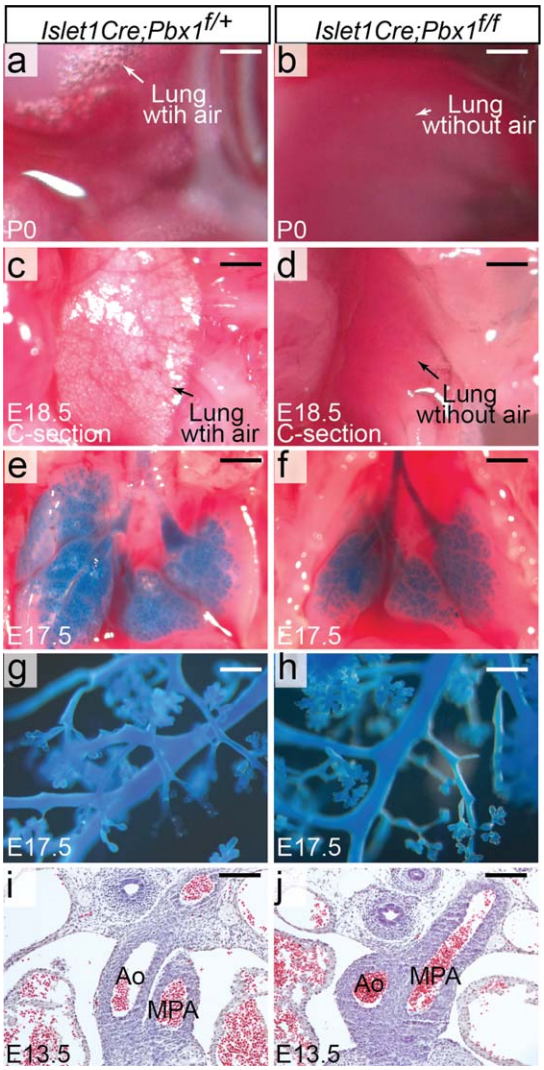


FIG. 2. The lungs of *Islet1Cre;Pbx1*$^{f/f}$ mice fail to expand at birth. (**a–d**) Air absorption in lung of control (*Islet1Cre;Pbx1*$^{f/+}$) and mutant (*Islet1Cre;Pbx1*$^{f/f}$) mice at P0 (a,b) and E18.5 after C-section (c,d). C-section, caesarean section. Size bars for (a,b): 2 mm. Size bars for (c,d): 3 mm. (**e–h**) Airway casting of control (*Islet1Cre;Pbx1*$^{f/+}$; e,g) and mutant (*Islet1Cre;Pbx1*$^{f/f}$; f,h) mice at E17.5. Size bars for (e,f): 4 mm. Size bars for (g,h): 50 μm. (**i,j**) HE staining of control (*Islet1Cre;Pbx1*$^{f/+}$; i) and mutant (*Islet1Cre;Pbx1*$^{f/f}$; j) mice at E13.5. Ao, aorta; MPA, main pulmonary artery. Size bars: 200 μm.

2000), we tested the expression of genes encoding the surfactant proteins in the lungs. By RT–qPCR, we found that the expression of surfactant genes—*Spa*, *Spb*, *Spc*, and *Spd*—was reduced by 36–72% in the lungs of *Islet1-Cre;Pbx1*$^{f/f}$ mice (Fig. 3h). Western blot of these mutant embryos also showed a dramatically reduction of all surfactant proteins in *Pbx1* deleted lungs (Fig. 3i). Such compound reduction of all surfactants provides an explanation for the failure of postnatal alveolar expansion in the mutant mice.

Pbx1 is Essential for *Fgf10* Expression in the Lung Mesenchyme

We next questioned how Pbx1 expressed in the lung mesenchyme controlled the surfactant genes that are expressed by alveolar type II cells (Mendelson, 2000). During lung development, the growth factors of Fgf7 and Fgf10 are secreted from the mesenchyme to activate the proliferation of alveolar type II cells that express surfactant genes (*Spa*, *Spb*, *Spc*, and *Spd*; Cardoso *et al.*, 1997; Chelly *et al.*, 1999; Hyatt *et al.*, 2004; Mason *et al.*, 2002; Ramasamy *et al.*, 2007). Furthermore, localized Fgf10 expression in the mesenchyme is essential for the differentiation of lung epithelial progenitors *in vivo* (Volckaert *et al.*, 2013). In addition, the semaphorin-3a protein (Sema3a) is expressed by the mesenchyme to negatively regulate the size and shape of the emerging saccules (Cirulli and Yebra, 2007; Ito *et al.*, 2000). Given that *Fgf7*, *Fgf10*, and *Sema3a* are expressed in the lung mesenchyme (Cirulli and Yebra, 2007), we tested if the mesenchymal Pbx1 was required for their expression. By RT–qPCR, we found that *Fgf10* showed a 65% decrease of mRNA level ($P < 0.0001$) in mutant lungs; the other two genes had no changes (Fig. 4a). Western blot and immunostaining of *Islet1Cre;Pbx1*$^{f/f}$ embryos showed a severe reduction of Fgf10 proteins in *Pbx1* deleted lungs (Fig. 4b–d). These findings suggest that Pbx1 in the lung mesenchyme is essential for activating *Fgf10* expression in the lungs.

Pbx1 Cooperates with Hox and Meis Proteins to Transcriptionally Activate *Fgf10*

To test whether Pbx1 directly regulated *Fgf10* expression, we used chromatin–immunoprecipitation and quantitative PCR (ChIP–qPCR) to examine the binding of Pbx1 to the *Fgf10* promoter. With sequence analysis by TFSEARCH software (www.cbrc.jp), we identified 5 regions (F1–F5) that contained potential Pbx1 binding sites in the proximal 4 kb promoter of mouse *Fgf10* (Fig. 4e). ChIP–qPCR analyses of mouse E16.5 lungs (about 10 embryonic lungs were required for each ChIP experiments) using anti-Pbx1 antibody (Chang *et al.*, 1997, 2008) showed that Pbx1 proteins were enriched within the F1–F5 regions, but not a negative control region (NC), of *Fgf10* (Fig. 4f). There was also no enrichment of Pbx1 in a negative control site on the *Admst1* promoter (Stankunas *et al.*, 2008a; Fig. 4f). The ChIP assay therefore demonstrates that Pbx1 proteins are associated with Pbx1 binding sites in the proximal promoter of *Fgf10* in the lungs.

To study the transcriptional activity of Pbx1 on the *Fgf10* promoter, we cloned the full-length proximal *Fgf10* promoter (−3905, +246) into a luciferase reporter plasmid pREP4 (Hang *et al.*, 2010). The *Fgf10* reporter plasmid and Pbx1, Meis1, and/or HoxB4 expressing plasmids (Chang *et al.*, 1995, 1996, 2008)

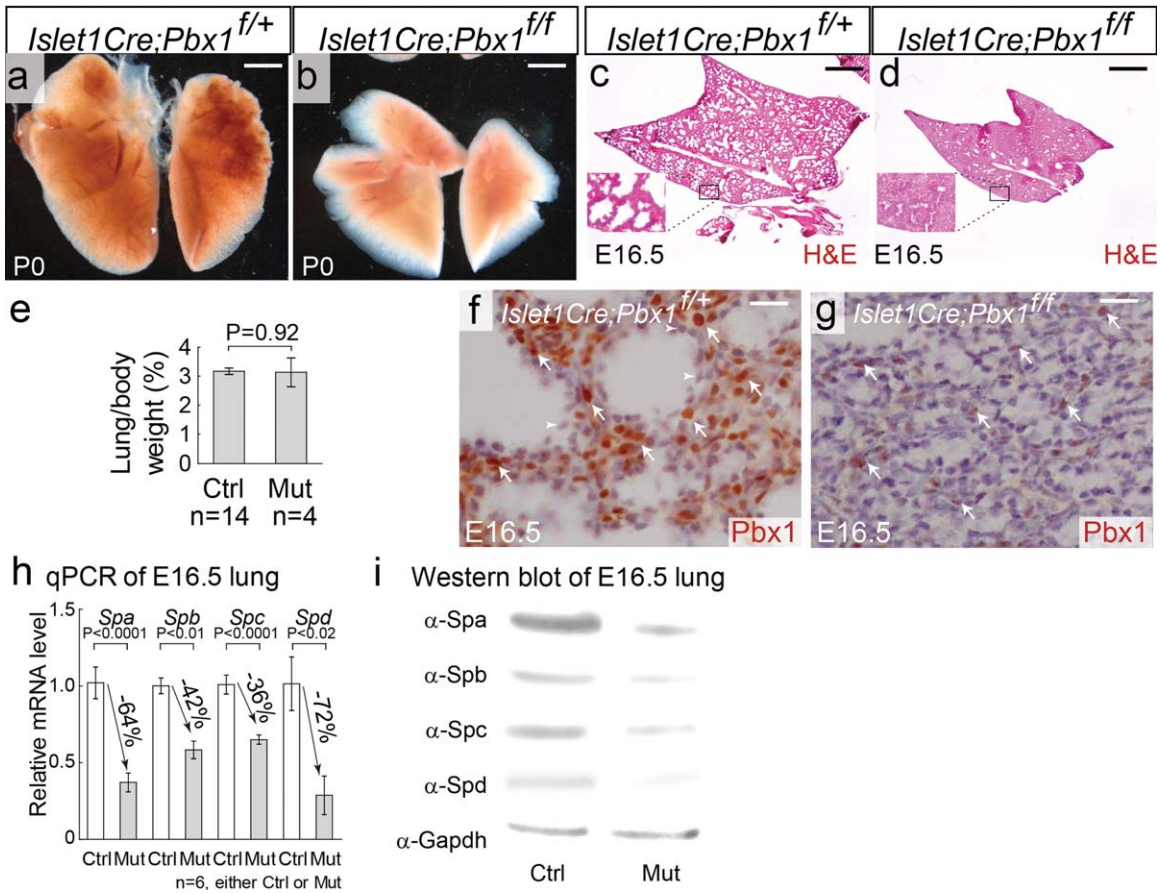


FIG. 3. Pbx1 is essential for lung development. (**a**) Lung morphology of control (*Islet1Cre;Pbx1*$^{f/+}$) and (**b**) mutant (*Islet1Cre;Pbx1*$^{f/f}$) mice at P0. Size bars: 2 mm. (**c**) H&E stained transverse lung sections of control (*Islet1Cre;Pbx1*$^{f/+}$) and (**d**) mutant (*Islet1Cre;Pbx1*$^{f/f}$) mice at E16.5. H&E, hematoxylin and eosin. Size bars: 700 μm. (**e**) Quantification of lung weight and body weight ratio of control (*Islet1Cre;Pbx1*$^{f/+}$) and mutant (*Islet1Cre;Pbx1*$^{f/f}$) mice at E16.5. (**f**) Pbx1 immunostaining (brown) in transverse lung sections of control (*Islet1Cre;Pbx1*$^{f/+}$) and (**g**) mutant (*Islet1Cre;Pbx1*$^{f/f}$) mice at E16.5. Arrow: Pbx1 staining. Arrow head: epithelial cells. Counterstain: hematoxylin. Size bars: 50 μm. (**h**) Quantitation of surfactant proteins (*Spa*, *Spb*, *Spc*, and *Spd*) mRNA expression levels by RT–qPCR in control and mutant lungs at E16.5. *P*-value: calculated by Student t-test. Error bar: standard error of the mean. (**i**) Western blot analysis of the protein levels of Spa, Spb, Spc, and Spd in lung samples prepared from control or mutant mice at E16.5. Gapdh was used as the internal control.

were cotransfected into a human lung carcinoma cell line H1299 for transient transactivation of reporter activities. By measuring the luciferase activity driven by *Fgf10* reporter, we found that Pbx1 modestly increase the *Fgf10* promoter activity by 30%. However, in the presence of Pbx1's partners Meis1 and HoxB4 (Chang *et al.*, 1995, 1997), Pbx1 enhanced the *Fgf10* promoter activity by 8.14-fold (Fig. 4g), suggesting synergistic interactions among Pbx, Meis, and Hox transcription factors in the activation of *Fgf10*. These results, together with the ChIP analysis, indicate a direct transcriptional activation of *Fgf10* by Pbx1 and its cofactors.

DISCUSSION

By using *Islet1Cre* to delete *Pbx1*, we serendipitously found that Pbx1 is essential in the lung mesenchyme to transcriptionally activate the expression of *Fgf10*, which encodes an essential factor for the differentiation of epithelial progenitors (Volckaert *et al.*, 2013; Fig. 4h). Our studies suggest that Pbx1 cooperates with Meis and Hox proteins to control *Fgf10* expression in the lung mesenchyme. Disruption of *Pbx1* in the lung mesenchyme interrupts the Pbx1–Fgf10 pathway, causing failure of alveolar expansion at birth and subsequent neonatal lethality.

Although Islet1 is a marker of cardiac progenitor cells derived from the second heart field (Cai *et al.*, 2003), Islet1 is not restricted to second heart field progenitors. It also labels the neural crest and lungs, including the lung mesenchymal cells. The expression of *Islet1* in those tissues has been documented by lineage tracing using *Islet1Cre* with a *Rosa26*^{lacz} reporter allele (Engleka *et al.*, 2012; High *et al.*, 2009; Yu *et al.*, 2010).

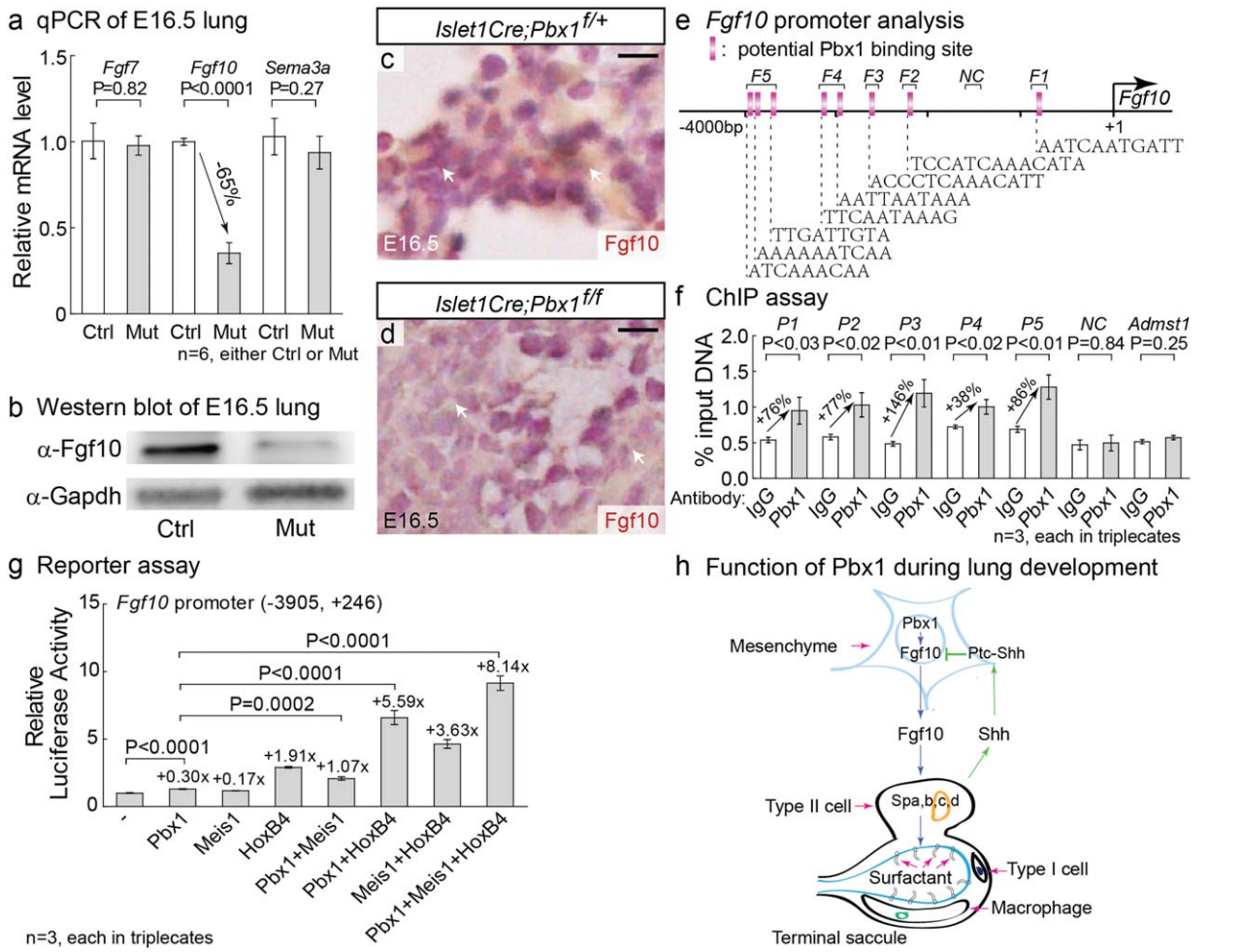


FIG. 4. Pbx1 activates Fgf10 in the lung mesenchyme during lung development. (**a**) Quantitation of *Fgf7*, *Fgf10*, and *Sema3a* mRNA expression levels by RT–qPCR in control and mutant lungs at E16.5. *P*-value: calculated by Student t-test. Error bar: standard error of the mean. (**b**) Western blot analysis of Fgf10 protein level in lung samples prepared from control or mutant mice at E16.5. Gapdh was used as the internal control. (**c**) Fgf10 immunostaining (brown) in transverse lung sections of control (*Islet1Cre;Pbx1*$^{f/+}$) and (**d**) mutant (*Islet1-Cre;Pbx1*$^{f/f}$) mice at E16.5. Arrow: Fgf10 staining. Counterstain: hematoxylin. Size bars: 20 μm. (**e**) Schematic of the *Fgf10* locus for potential Pbx1 binding sites by sequence analysis. Potential Pbx1 binding regions (brackets *F1–F5*) and a negative control region (bracket *NC*) were further analyzed by ChIP. The DNA positions are denoted relative to the transcriptional start site (+1). (**f**) qPCR quantification of control IgG and Pbx1 antibodies-immunoprecipitated chromatin from E16.5 mouse embryonic lung samples using primers targeting *Fgf10* promoter (*F1–F5, NC*) and *Admst1* promoter. Signals were standardized to percentage of input DNA. *P*-value: calculated by Student t-test. Error bar: standard error of the mean. (**g**) Luciferase reporter assays of *Fgf10* promoter (−3905, +246) in H1299 cells cotransfected with plasmids expressing the indicated transcription factors. *P*-value: calculated by Student t-test. Error bar: standard error of the mean. (**h**) Working model of how Pbx1 in lung mesenchymal cells activates the expression of Fgf10. Pbx1 in the mesenchymal cells may form a negative feedback loop with Shh secreted by alveolar cells to control the level of Fgf10 in the developing lung.

Furthermore, the unexpected normal heart morphogenesis of *Islet1Cre Pbx1*$^{f/f}$ mice could be the result of the following factors. First, *Pbx1* may not function in the secondary heart field for regulating aortopulmonary septation. Pbx1 may work in cell types not labeled by *Islet1Cre*. Second, because a time delay exists between Cre expression and gene deletion, the deletion of *Pbx1* by *Islet1Cre* may occur after the major actions of Pbx1 in cardiac progenitor cells have completed, thereby generating no cardiac phenotype. Further studies using other Cre lines to define the site and the time of Pbx1 action for heart development are essential to understand the cardiac function of Pbx1.

In embryos, active chloride and fluid secretions across the airway epithelium are essential for normal lung morphogenesis (Blaisdell *et al.*, 2000). The compacted lungs of *Islet1Cre Pbx1*$^{f/f}$ embryos suggest that Pbx1 may also regulate chloride and fluid secretion during lung development. Further studies are needed to elucidate the mechanism. In contrast, the neonates require surfactants to allow the expansion of alveoli at birth. Surfactant proteins are lipoproteins that serve a key role in the

adsorption and spreading of fluid at the alveolar air–liquid interface (Veldhuizen *et al.*, 1998). Surfactants include four proteins, Spa, Spb, Spc, and Spd (Mendelson, 2000), the abnormalities of which in mice or patients can cause RDS, which affects ~1% of the newborn and is the leading cause of death in preterm infants (Ballard *et al.* 2003; Clements and Avery 1998; Rodriguez *et al.*, 2002). The lungs of *Spa−/−* mice were susceptible to infections (Ikegami *et al.*, 1998), and human SPA was shown to be anti-inflammatory (Lee *et al.*, 2010), suggesting that Spa is important for immune defense within the alveoli. Furthermore, both *Spb−/−* mice (Clark *et al.*, 1995) and SPB-deficient infants (Nogee *et al.*, 1993) displayed RDS phenotype. Interestingly, with the Spb deficiency, Spc is abnormally processed and immature, resulting in an additional reduction of surfactant function with consequent severe RDS phenotype (Vorbroker *et al.*, 1995). These studies indicate the necessity of Spb and Spc for alveolar function. In contrast, *Spd−/−* mice developed a progressive accumulation of surfactant lipids within the alveoli, suggesting a critical role of Spd in surfactant homeostasis (Botas *et al.*, 1998).

The expression of surfactant genes in the developing lungs is regulated by many factors, including cyclic AMP, glucocorticoids, retinoids, insulin, growth factors, and cytokines (EGF, TGF, TNF, IFN, and Interleukin) (Mendelson, 2000; Mendelson *et al.*, 1998). Furthermore, Fgf10 is critical for the proliferation of alveolar type II cells that secrete Spa, Spb, Spc, and Spd (Mason *et al.*, 2002; Ramasamy *et al.*, 2007), the reprogramming of mouse tracheal epithelium to express Spc *in vitro* (Hyatt *et al.*, 2004), as well as the regulation of lung epithelial progenitors *in vivo* (Volckaert *et al.*, 2013). The expression of Fgf10 is regulated by both Pbx1 (this studies) and Shh (Bellusci *et al.*, 1997b; Murone *et al.*, 1999; Pepicelli *et al.*, 1998). Pbx1 in the mesenchymal cells is required for Fgf10 expression, whereas Shh secreted from the alveolar epithelium binds to its receptor Patched on the mesenchymal cells to suppress Fgf10 expression. Absence of Pbx1 in mesenchymal cells or overexpression of *Shh* driven by *Spc* enhancer/promoter in embryonic lungs resulted in Fgf10 reduction, compaction of terminal saccules, and neonatal lethality (this studies; Bellusci *et al.*, 1997a). In contrast to mice lacking Pbx1, mouse embryos deficient in Shh had enhanced expression of Fgf10 in the lung (Pepicelli *et al.*, 1998). Shh and Pbx1 therefore appear to form a negative feedback loop between alveolar and mesenchymal cells to regulate the level of Fgf10 in the developing lung (Fig. 4h). Further analyses of the interactions between Shh signaling and the Pbx–Fgf10 pathway will be crucial to provide a deeper insight into the molecular mechanisms underlying lung mesenchymal and epithelial development.

Our studies have clinical implications. Mice with deletion of *Pbx1* in the lung mesenchyme can provide an animal model to study the mechanisms that underlies RDS. Recently, two single nucleotide genetic mutations in *PBX1* were observed in women with Mullerian duct abnormalities (Ma *et al.*, 2011). It will be interesting for future investigations to determine whether *PBX1* mutations could also underlie RDS in some patients.

METHODS

Mice

Pbx1$^{f/f}$ (Koss *et al.*, 2012) and *Islet1Cre* (Cai *et al.*, 2003; Yang *et al.*, 2006) mice have been described previously. The date of observing a vaginal plug was set as E0.5, and embryonic development was confirmed by ultrasonography before sacrificing pregnant mice (Chang *et al.*, 2003). The use of mice for studies is in compliance with the regulations of Stanford University and National Institute of Health.

Histology, Immunostaining, and Airway Casting

Histological analysis and immunostaining were performed as described (Chang *et al.*, 2004, 2008). All these procedures were performed on 7 μm paraffin sections of the lungs which were freshly prefixed by 4% PFA. Hematoxylin and eosin (H&E) stain was performed according to standard protocols. The following primary antibodies were used for immunostaining: anti-Pbx1 antibody (41.1, a gift from Dr. M. Cleary, dilution 1:100), anti-Fgf10 (AF6224, R&D systems, dilution 1:40). Airway casting with an acrylic resin (Batson no. 17) containing blue dye (Methyl Methacrylate Casting Kit, Polyscience) was performed as described (Chang *et al.*, 2008).

RNA Isolation and Reverse Transcription–Quantitative Polymerase Chain Reaction (RT–qPCR)

Total RNA was extracted using TRIZOL followed by RNase-free DNase I treatment (NEB) to avoid genomic contamination. Total RNA (1 μg for each sample) was reverse transcribed by iScript (BioRad, Hercules, CA) and quantitative real time PCR analysis was performed using SYBR Green supermix (BioRad, Hercules, CA) or Taqman reagents (Applied Biosciences) according to manufacturer's recommendations. Murine Pbx1 and Gapdh were by Taqman probes (Applied Biosciences, Foster City, CA). The other primer sets used were tested to be quantitative:

- Murine *Tf2b* F: CTCTGTGGCGGCAGCAGCTATTT
- Murine *Tf2b* R: CGAGGGTAGATCAGTCTGTAGGA
- Murine *Spa* F: GAGAGCCTGGAGAAAGGGGG
- Murine *Spa* R: GGATCCTTGCAAGCTGAGGA

- Murine *Spb* F: CCAAGTGCTTGATGTCTACC
- Murine *Spb* R: CTGGATTCTGTTCTGGCTTA
- Murine *Spc* F: GTAGCAAAGAGGTCCTGATG
- Murine *Spc* R: CCTACAATCACCACGACAA
- Murine *Spd* F: GAGCCTGACAAACAGAGGT
- Murine *Spd* R: CTGTACAAGCAAGACAAGCA
- Murine *Fgf7* F: GAAGACTGTTCTGTCGCACCC
- Murine *Fgf7* R: AACTGCCACGGTCCTGATTTC
- Murine *Fgf10* F: AAGCCATCAACAGCAACTAT
- Murine *Fgf10* R: ATTGTGCTGCCAGTTAAAAG
- Murine *Sema3a* F: TGCTCACAGAGATGGTCCCAA
- Murine *Sema3a* R: TGTGGAGTCAAATCCGCCAAA

Chromatin Immunoprecipitation–Quantitative PCR (ChIP–qPCR) The ChIP procedure was described previously (Hang *et al*., 2010; Stankunas *et al*., 2008a). Chromatin from E16.5 lungs (about 10 embryos for each ChIP) was sonicated to an average length between 200 and 500 base pairs, and immunoprecipitated using anti-Pbx1 antibody (41.1, a gift from Dr. M. Cleary), or control IgG; 5% of the pull down was taken for subsequent qPCR analysis. ChIP–qPCR signals of individual ChIP reaction was standardized to its own input qPCR signals. PCR primers for the potential Pbx1 binding regions on *Fgf10* promoter: *F1-F* GTC CCTGATTTCATTTGCGCC, *F1-R* CTCGCTTCCGTTG CTGAAGTA; *F2-F* GGGAGTGTTGGGCTGAAGAAG, *F2-R* AGTTTGGGGTTTCTTTACACTGGA; *F3-F* ATGT-CAGCTTTTCCTTTGGGCA, *F3-R* GCAATGTTTGA GGGTTCCCGA; *F4-F* CGTCGAATTTAACAGCAGCT-TACC, *F4-R* GCTGTCTGTCTCTTTCATCCGA; *F5-F* TT CCTATGGCTGGGTTGCCTA, *F5-R* ATCAACCACGTTG-GAGCTCAG. Control primers for the region without potential Pbx1 binding site on *Fgf10* promoter: *NC-F* CCAGAACACAGATGTCTAACT, *NC-R* TGAGTACCAGA-GATCATTTCC. Control primers for promoter of *Admst1* were described previously (Stankunas *et al*., 2008a). The DNA positions are denoted relative to the transcriptional start site (+1).

Cloning and Luciferase Reporter Assay

The reporter assays were previously described (Chang *et al*., 2008; Hang *et al*., 2010; Wu *et al*., 2007). Full length of intergenic *Fgf10* promoter which span (−3905, +246) was cloned into pREP4-Luc reporter plasmid (Liu *et al*., 2001). These constructs were then transfected into a human lung carcinoma cell line H1299 with lipofectamine 2000 (Invitrogen, Carlsbad, CA) along with pREP7-RL as a transfection efficiency control, Pbx1, Meis1, and HoxB4 expression vectors (Chang *et al*., 2008) with the appropriate empty vector control. Luciferase activity was measured and normalized to a cotransfected Renilla luciferase construct using the Dual-Luciferase Reporter System (Promega, Madison, WI).

Western Immunoblot Analysis

Mouse E16.5 lungs were collected and washed once with ice-cold PBS and lysed with SDS buffer. After boiling, the supernatants were collected. The blots were reacted with antibodies of anti-Spa (ab115791, Abcam, dilution 1:500), anti-Spb (ab3282, Abcam, dilution 1:100), anti-Spc (ab90716, Abcam, dilution 1:1000), anti-Spd (ab17781, Abcam, dilution 1:10000), anti-Fgf10 (AF6224, R&D systems, dilution 1:100), anti-Gapdh (G9545, Sigma, dilution 1:10000), followed by HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Chemiluminescence was detected with ECL Western blot detection kits (GE).

ACKNOWLEDGMENTS

The authors thank Dr. Sylvia Evans for providing *Islet1Cre* mice; Dr. Michael Cleary for discussion and advice; Drs. Marlene Rabinovitch and Richard D. Bland for reading of this manuscript and advice.

LITERATURE CITED

Ballard PL, Merrill JD, Godinez RI, Godinez MH, Truog WE, Ballard RA. 2003. Surfactant protein profile of pulmonary surfactant in premature infants. Am J Respir Crit Care Med 168:1123–1128.

Beers MF, Hamvas A, Moxley MA, Gonzales LW, Guttentag SH, Solarin KO, Longmore WJ, Nogee LM, Ballard PL. 2000. Pulmonary surfactant metabolism in infants lacking surfactant protein B. Am J Respir Cell Mol Biol 22:380–391.

Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL. 1997a. Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 124:53–63.

Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL. 1997b. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 124:4867–4878.

Blaisdell CJ, Edmonds RD, Wang XT, Guggino S, Zeitlin PL. 2000. pH-regulated chloride secretion in fetal lung epithelia. Am J Physiol Lung Cell Mol Physiol 278:L1248–1255.

Botas C, Poulain F, Akiyama J, Brown C, Allen L, Goerke L, Clements J, Carlson E, Gillespie AM, Epstein C, Hawgood S. 1998. Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. Proc Natl Acad Sci USA 95:11869–11874.

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL. Chen J, Evans S 2003. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 5:877–889.

Cardoso WV. 2001. Molecular regulation of lung development. Annu Rev Physiol 63:471–494.

Cardoso WV, Itoh A, Nogawa H, Mason I, Brody JS. 1997. FGF-1 and FGF-7 induce distinct patterns of growth and differentiation in embryonic lung epithelium. Dev Dyn 208:398–405.

Chang CP, Brocchieri L, Shen WF, Largman C, Cleary ML. 1996. Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. Mol Cell Biol 16:1734–1745.

Chang CP, Chen L, Crabtree GR. 2003. Sonographic staging of the developmental status of mouse embryos in utero. Genesis 36:7–11.

Chang CP, Jacobs Y, Nakamura T, Jenkins NA, Copeland NG, Cleary ML. 1997. Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. Mol Cell Biol 17:5679–5687.

Chang CP, Neilson JR, Bayle JH, Gestwicki JE, Kuo A, Stankunas K, Graef IA, Crabtree GR. 2004. A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis. Cell 118:649–663.

Chang CP, Shen WF, Rozenfeld S, Lawrence HJ, Largman C, Cleary ML. 1995. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev 9:663–674.

Chang CP, Stankunas K, Shang C, Kao SC, Twu KY, Cleary ML. 2008. Pbx1 functions in distinct regulatory networks to pattern the great arteries and cardiac outflow tract. Development 135:3577–3586.

Chelly N, Mouhieddine-Gueddiche OB, Barlier-Mur AM, Chailley-Heu B, Bourbon JR. 1999. Keratinocyte growth factor enhances maturation of fetal rat lung type II cells. Am J Respir Cell Mol Biol 20:423–432.

Cirulli V, Yebra M. 2007. Netrins: Beyond the brain. Nat Rev Mol Cell Biol 8:296–306.

Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stripp BR, Weaver TE, Whitsett JA. 1995. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. Proc Natl Acad Sci USA 92:7794–7798.

Clements JA, Avery ME. 1998. Lung surfactant and neonatal respiratory distress syndrome. Am J Respir Crit Care Med 157:S59–66.

Engleka KA, Manderfield LJ, Brust RD, Li L, Cohen A, Dymecki SM, Epstein JA. 2012. Islet1 derivatives in the heart are of both neural crest and second heart field origin. Circ Res 110:922–926.

Gower WA, Nogee LM. 2011. Surfactant dysfunction. Paediatr Respir Rev 12:223–229.

Halliday HL. 2008. Surfactants: Past, present and future. J Perinatol 28 Suppl 1:S47–56.

Hang CT, Yang J, Han P, Cheng HL, Shang C, Ashley E, Zhou B, Chang CP. 2010. Chromatin regulation by Brg1 underlies heart muscle development and disease. Nature 466:62–67.

Hawgood S. 2004. Surfactant protein B: Structure and function. Biol Neonate 85:285–289.

High FA, Jain R, Stoller JZ, Antonucci NB, Lu MM, Loomes KM, Kaestner KH, Pear WS, Epstein JA. 2009. Murine Jagged1/Notch signaling in the second heart field orchestrates Fgf8 expression and tissue-tissue interactions during outflow tract development. J Clin Invest 119:1986–1996.

Hyatt BA, Shangguan X, Shannon JM. 2004. FGF-10 induces SP-C and Bmp4 and regulates proximal-distal patterning in embryonic tracheal epithelium. Am J Physiol Lung Cell Mol Physiol 287:L1116–1126.

Ikegami M, Korfhagen TR, Whitsett JA, Bruno MD, Wert SE, Wada K, Jobe AH. 1998. Characteristics of surfactant from SP-A-deficient mice. Am J Physiol 275: L247–254.

Ito T, Kagoshima M, Sasaki Y, Li C, Udaka N, Kitsukawa T, Fujisawa H, Taniguchi M, Yagi T, Kitamura H, Goshima Y. 2000. Repulsive axon guidance molecule Sema3A inhibits branching morphogenesis of fetal mouse lung. Mech Dev 97:35–45.

Koss M, Bolze A, Brendolan A, Saggese M, Capellini TD, Bojilova E, Boisson B, Prall OW, Elliott DA, Solloway M, Lenti E, Hidaka C, Chang CP, Mahlaoui N, Harvey RP, Casanova JL, Selleri L. 2012. Congenital asplenia in mice and humans with mutations in a Pbx/Nkx2–5/p15 module. Dev Cell 22: 913–926.

Lee DC, Romero R, Kim CJ, Chaiworapongsa T, Tarca AL, Lee L, Suh YL, Mazaki-Tovi S, Vaisbuch E, Mittal P, Draghici S, Erez O, Kusanovic JP, Hassan SS, Kim JS. 2010. Surfactant protein-A as an anti-inflammatory component in the amnion: Implications for human pregnancy. J Immunol 184:6479–6491.

Liu R, Liu H, Chen X, Kirby M, Brown PO, Zhao K. 2001. Regulation of CSF1 promoter by the SWI/SNF-like BAF complex. Cell 106:309–318.

Ma J, Qin Y, Liu W, Duan H, Xia M, Chen ZJ. 2011. Analysis of PBX1 mutations in 192 Chinese women with Mullerian duct abnormalities. Fertil Steril 95:2615–2617.

Mason RJ, Lewis MC, Edeen KE, McCormick-Shannon K, Nielsen LD, Shannon JM. 2002. Maintenance of surfactant protein A and D secretion by rat alveolar type II cells in vitro. Am J Physiol Lung Cell Mol Physiol 282:L249–258.

Mendelson CR. 2000. Role of transcription factors in fetal lung development and surfactant protein gene expression. Annu Rev Physiol 62:875–915.

Mendelson CR, Gao E, Li J, Young PP, Michael LF, Alcorn JL. 1998. Regulation of expression of surfactant protein-A. Biochim Biophys Acta 1408: 132–149.

Murone M, Rosenthal A, de Sauvage FJ. 1999. Sonic hedgehog signaling by the patched-smoothened receptor complex. Curr Biol 9:76–84.

Nkadi PO, Merritt TA, Pillers DA. 2009. An overview of pulmonary surfactant in the neonate: Genetics, metabolism, and the role of surfactant in health and disease. Mol Genet Metab 97:95–101.

Nogee LM. 2004. Genetic mechanisms of surfactant deficiency. Biol Neonate 85:314–318.

Nogee LM, de Mello DE, Dehner LP, Colten HR. 1993. Brief report: Deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. N Engl J Med 328:406–410.

Pepicelli CV, Lewis PM, McMahon AP. 1998. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. Curr Biol 8:1083–1086.

Ramasamy SK, Mailleux AA, Gupte VV, Mata F, Sala FG, Veltmaat JM, Del Moral PM, De Langhe S, Parsa S, Kelly LK, Kelly R, Shia W, Keshet E, Minoo P, Warburton D, Bellusci S. 2007. Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. Dev Biol 307: 237–247.

Rodriguez RJ, Martin RJ, Fanaroff AA. 2002. Respiratory distress syndrome and its management (7th ed., pp. 1001–1011). St. Louis, MO: Mosby.

Stankunas K, Hang CT, Tsun ZY, Chen H, Lee NV, Wu JI, Shang C, Bayle JH, Shou W, Iruela-Arispe ML, Chang CP. 2008a. Endocardial Brg1 represses ADAMTS1 to maintain the microenvironment for myocardial morphogenesis. Dev Cell 14:298–311.

Stankunas K, Shang C, Twu KY, Kao SC, Jenkins NA, Copeland NG, Sanyal M, Selleri L, Cleary ML, Chang CP. 2008b. Pbx/Meis deficiencies demonstrate multigenetic origins of congenital heart disease. Circ Res 103:702–709.

Veldhuizen R, Nag K, Orgeig S, Possmayer F. 1998. The role of lipids in pulmonary surfactant. Biochim Biophys Acta 1408:90–108.

Volckaert, T, Campbell A, Dill E, Li C, Minoo P, De Langhe S. 2013. Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. Development 140:3731–3742.

Vorbroker DK, Profitt SA, Nogee LM, Whitsett JA. 1995 Aberrant processing of surfactant protein C in hereditary SP-B deficiency. Am J Physiol 268:L647–656.

Whitsett JA, Weaver TE. 2002. Hydrophobic surfactant proteins in lung function and disease. N Engl J Med 347:2141–2148.

Wu H, Kao SC, Barrientos T, Baldwin SH, Olson EN, Crabtree GR, Zhou B, Chang CP. 2007. Down syndrome critical region-1 is a transcriptional target of nuclear factor of activated T cells-c1 within the endocardium during heart development. J Biol Chem 282:30673–30679.

Yang L, Cai CL, Lin L, Qyang Y, Chung C, Monteiro RM, Mummery CL, Fishman GI, Cogen A, Evans S. 2006. Isl1Cre reveals a common Bmp pathway in heart and limb development. Development 133:1575–1585.

Yu S, Poe B, Schwarz M, Elliot SA, Albertine KH, Fenton S, Garg V, Moon AM. 2010. Fetal and postnatal lung defects reveal a novel and required role for Fgf8 in lung development. Dev Biol 347:92–108.

Yurdakok M. 2004. Inherited disorders of neonatal lung diseases. Turk J Pediatr 46:105–114.