*ERJ Express*. Published on February 12, 2009 as doi: 10.1183/09031936.00157108

# Chronological expression of Ciliated Bronchial Epithelium 1 during pulmonary development.

Hans Michael Haitchi[1], Hajime Yoshisue[1], Anna Ribbene[1,3], Susan J. Wilson[1], John W. Holloway[1,2], Fabio Bucchieri[1,3], Neil A. Hanley[2], David I. Wilson[2], Giovanni Zummo[3], Stephen T. Holgate[1], Donna E. Davies[1]

[1]Infection, Inflammation and Repair Division and [2]Centre for Human Development, Stem Cells and Regeneration, Human Genetics Division, School of Medicine, University of Southampton, Southampton General Hospital, Southampton, United Kingdom, [3]Human Anatomy Section, University of Palermo, Palermo, Italy

***Address for correspondence**:

Professor Donna E Davies
Division of Infection, Inflammation and Repair,
School of Medicine,
University of Southampton,
Southampton General Hospital,
Tremona Road,
Southampton, SO16 6YD,
United Kingdom
Tel.: +44 (0)23 8079 8523
Fax: +44 (0)23 8070 1771
Email:donnad@soton.ac.uk

Running title: *Cbe1*/*CBE1* expression during lung development.

Word count: 3000

**ABSTRACT:**

*Ciliated Bronchial Epithelium* (*CBE*) *1* is a novel gene, expressed in ciliated cells. As cilia are important during embryogenesis, we have characterized the murine homologue of *CBE1* (*Cbe1*) and compared its temporal expression during murine and human lung development.

*Cbe1* cDNA was cloned and characterised using sequencing, standard PCR and Western blotting. Mouse and human embryonic/fetal lungs (HELs) were harvested for mRNA analysis and protein localisation *in vivo* and *in vitro* using RT-PCR and immunohistochemistry.

Cbe1 amino acid sequence was >75% identical with CBE1 and its alternative splicing and tissue distribution were highly conserved. Pulmonary expression of *Cbe1* mRNA was increased at embryonic day 16 (E16), one day later than *Foxj1*, consistent with a role in ciliogenesis. In HELs, *CBE1* mRNA was detectable at 8-9 weeks post conception and increased in explant culture. CBE1 protein expression was weak at 10 weeks post conception but strong at 12.3 weeks post conception, in parallel with cilia formation. Additionally, *Cbe1* mRNA was expressed at E11 (4-5 weeks post conception in HELs) in the absence of *Foxj1*, implying a distinct role in early development.

Chronological regulation of *CBE1/Cbe1* expression during pulmonary differentiation suggests involvement in ciliogenesis, with an additional role during early lung development.

**Keywords:** *CBE1*, Ciliogenesis, Epithelium, *FOXJ1*, Embryonic/Fetal Lung Development,

## INTRODUCTION

Cilia are finger-like appendages that are microtubule (MT)-based organelles, and are classified according to their MT components as 9+2 (motile) and 9+0 (primary) cilia. Airway is the archetypal tissue containing motile cilia. The ciliated cells in the tracheal and bronchial epithelium of the lower airways play a pivotal role in propelling mucus secretions towards the pharynx (1;2). Although the molecular mechanisms of epithelial ciliogenesis have not been fully investigated, the transcription factor forkhead box factor (*FOX*) *J1* (hepatocyte nuclear factor-3/forkhead homologue 4, *HFH-4*) is closely involved in ciliogenesis. Targeted disruption of the *Foxj1* gene in mice results in an absence of airway cilia and *situs inversus* (3;4), suggesting that *Foxj1* is important not only for the differentiation of airway epithelium but also for the normal positioning of internal organs. However, forced over expression of *Foxj1* in undifferentiated airway epithelial does not induce formation of ciliated cells (5), implying that *Foxj1* alone is not sufficient for the development of cilia and that ciliogenesis requires other different transcription factors that have yet to be characterised.

Recently we characterised a novel gene *Ciliated Bronchial Epithelium* (*CBE*) *1*, which was initially identified as a differentially represented gene in cDNA libraries derived from asthmatic and normal bronchial biopsies (6). Although its predicted amino acid sequence has no similarity to known proteins, expression of CBE1 is strongly associated with ciliated

epithelial cells both in bronchial and nasal tissues. Importantly, immunostaining was observed intracellularly, but not within the ciliary structure, suggesting that CBE1 does not constitute a component of cilia. Expression studies showed that CBE1 is localized to the nuclear or perinuclear regions of cells, implying that CBE1 might be a nucleocytoplasmic shuttling protein, although no clear function for CBE1 has yet been described. Although we have shown strong induction of the *CBE1* mRNA during *in vitro* mucociliary differentiation of primary bronchial epithelial cells, its expression during lung development has not been investigated. In this study, we have characterised the mouse ortholog of *CBE1* (*Cbe1*), analysed chronological expression of *Cbe1* mRNA during pulmonary differentiation *in vivo* and compared this with *CBE1* mRNA and protein expression in human embryonic/fetal lung explants cultured *in vitro.*

## MATERIALS AND METHODS

*Cloning and characterization of Cbe1* cDNA

cDNA from adult mouse lung was amplified using primers specific for *Cbe1* and the products cloned and sequenced. cDNAs encoding open reading frame (ORF)1 and ORF2 were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and transfected into HEK293 cells. Isolation of cellular extracts, SDS-PAGE and Western blotting were performed as described previously (6). Detailed protocols are provided in the Online depository.

*Isolation of mouse lungs*

Mouse lungs were harvested at embryonic day (E) 11-19, postnatal day (P) 1 and 8, and adult mice (AM). Dissected biopsies were immediately homogenised in Trizol solution (Invitrogen) for RNA isolation (see below).

*Isolation of human fetal lungs and ex vivo differentiation*

Human fetal lung tissues were collected from women undergoing first trimester termination of pregnancy with informed written consent and ethical approval. Isolated tissues were staged and processed as described previously (7), or cultured *in vitro* at an Matrigel air-liquid interface (ALI) using Ultraculture serum free medium (Cambrex; Verviers, Belgium) for up to 18 days.

*Immunohistochemistry*

Human fetal lungs were processed into glycol methacrylate resin and 2μm sections cut and subjected to immunohistochemical analysis with immunoperoxidase detection using diaminobenzidine or 3-amino-9-ethylcarbazole as chromagens, as previously described (8). Affinity purified rabbit polyclonal antibody generated against CBE1 (6) was used at 2 μg/ml.

*RNA extraction and RT- PCR*

RNA samples were isolated using Trizol reagent according to the manufacturer's instructions and treated with RNase-free DNase I (Ambion; Huntingdon, UK) to remove any contaminating genomic DNA. cDNA was synthesized as previously described (6). For semi-quantitative PCR and nested PCR, cDNA was amplified using specific primers as described in the Online Depository. PCR products were separated in 1.5% agarose gel and visualised with ethidium bromide or Vistra Green (Amersham Biosciences; Amersham, UK). Reverse transcription quantitative PCR (RT-qPCR) was performed using an IcyclerIQ system (Bio-Rad; Hemel Hempstead, UK) (9). Relative expression levels were calculated using the ΔΔCT method. Specific primers, probes and experimental conditions are provided in the Online Depository. The results were expressed relative to either *Gapdh* or *ACTB* mRNA levels which were used as house keeping genes.

*Statistical analyses*

Statistical analysis was undertaken using the Mann-Whitney test implemented in SPSS (SPSS, Inc.; Chicago, IL). $P$ values $< 0.05$ were considered as significant.

## RESULTS

*Characterization of Cbe1 and its tissue distribution*

To compare the expression profile of *CBE1* mRNA with that of its rodent counterpart, we cloned and characterised the murine ortholog of *CBE1* (*Cbe1*). Basic local alignment search tool (BLAST) search using *CBE1* as a query easily identified a highly homologous mouse mRNA (accession number AK003742 in the GenBank database) which has not been fully annotated. Specific primers were designed based on the sequence AK003742, and the putative full-length cDNA of *Cbe1* was amplified by PCR, followed by sequencing analyses. Figure 1a shows the resulting consensus sequence of the cDNA of *Cbe1*, within which we found a small open reading frame (ORF) consisting of 126 amino acids. The first methionine codon of this ORF is preceded by an in-frame stop codon 42 bp upstream, and is flanked by a Kozak's consensus sequence (A/GXXatgG) (10), suggesting that it is the genuine translation initiation site. Given this, together with a putative polyadenylation signal (AATAAA) in the 3-untranslated region (fig. 1a), this is a full-length cDNA. Two out of twelve independent clones which were sequence analysed showed a 5-bp insertion at one of the splicing sites, resulting in a frame shift, in turn, generating another ORF (ORF2) consisting of 162 amino acids with a different carboxyl terminus. It is interesting that the way these splicing variants are generated is completely conserved between mice and human (6). BLAST search analyses has also identified another splicing variant (accession number XM355478) of *Cbe1*

harbouring a longer amino-terminus, like *CBE1*. Specific primers were designed to detect this variant by RT-PCR, clarifying that the longer form is not expressed in lung but is expressed in testis; the longer form also has two splicing variants with different carboxyl termini, due to the 5-bp insertion (fig. 1b).

The predicted amino acid sequence of ORF1 of Cbe1 is 75.4% identical to that of CBE1, whereas 78.4% identity is found for ORF2 (fig. 1c). Although both ORFs have no obvious similarity to known proteins, the two arginine residues which could serve as one of the nuclear localisation signals in CBE1 (6) are conserved.

We also undertook semi-quantitative RT-PCR to evaluate the tissue distribution of *Cbe1* mRNA in adult tissues. This showed that the long form was testis-specific, and that the short form was abundantly expressed in lung and testis (fig 1b), with relatively lower expression in brain and thymus, and no detectable expression in other tissues analysed (heart, liver, spleen, kidney; fig. 2a), This latter finding is in contrast with *CBE1* mRNA which is also expressed in heart and kidney (6), although we could detect *Cbe1* mRNA in embryonic heart using RT-qPCR (data not shown). These data suggest that expression of *Cbe1* mRNA is highly tissue-specific, and that its pattern is partially consistent with that of *CBE1*.

*Regulation of Cbe1 mRNA expression during mouse embryogenesis*

The amino acid sequences in the two synthetic peptide sequences which were used previously to generate anti-CBE1 antibodies were not completely conserved between CBE1 and Cbe1 (6), with 11 out of 14 amino acids being identical in one peptide and 10 out of 14 identical in the other peptide. As a result, recombinant ORF1 and ORF2 of Cbe1 expressed in HEK293 cells showed much lower reactivity to anti-CBE1 antibodies compared to recombinant CBE1, when analysed by Western blotting (fig. 2b). Therefore, we chose to use RT-qPCR analyses to investigate the chronological expression of *Cbe1* mRNA and other ciliogenesis-related genes during embryogenesis.

Figure 3a shows that transcription of *Foxj1* mRNA, which is closely involved in ciliogenesis, was switched on at E15 showing a 15.4±3.7-fold induction compared to the basal level at E14. *Foxj1* mRNA chronologically increased thereafter, up to 342±71-fold in adult mice. Expression of *Cbe1* mRNA increased 15.4±7.4-fold at E16 compared to the basal level at E14, which was later than *Foxj1*, and increased 447±99-fold in adult mice (fig 3b). In contrast, the expression profile of *Foxa1* and *Foxa2* mRNAs, forkhead transcription factors closely involved in the differentiation of bronchial epithelium (11;12), showed little change (*Foxa1*, fig 3c) or increased only 3-5 fold (*Foxa2*, fig 3d) from E11 to adult, consistent with a previous report that these transcription factors are expressed from E10.5 (13). *Tektin-1*

*(Tetk1)*, a gene encoding proteins which form filamentous polymers in the walls of ciliary and flagellar microtubules (14) , showed a 4.5±1.1 fold increase in mRNA expression going from E15 to E16, which was much less than for *Cbe1* or *Foxj1* mRNA (fig 3e). Although the expression profile and similar kinetics of *Cbe1* and *Foxj1* mRNA during the late pseudoglandular stage of lung development (fig 3f) are consistent with a role for Cbe1 in ciliogenesis, significantly greater expression of *Cbe1* mRNA was observed at E11 compared to E12-14, but this was not observed for *Foxj1* (fig 3a,b), suggesting that Cbe1 has a distinct function in early lung development.

*Expression of CBE1 in human embryonic/fetal lungs*

We analysed the expression of *CBE1* mRNA in human embryonic/fetal lungs using semi-quantitative RT-PCR; this showed a low but detectable amount of *CBE1* mRNA at 10 weeks post conception. In contrast, *FOXJ1* mRNA was consistently observed from 7 to 10 weeks post conception whereas *TEKT1* mRNA was not expressed at 10 weeks post conception (fig. 4a). In order to detect low copy numbers of *CBE1* and *TEKT1* mRNA, we performed nested PCR which revealed that *CBE1* mRNA was already present at 8 to 9 weeks post conception, whereas *TEKT1* mRNA was not detectable even with this highly sensitive method (fig. 4b). These results suggest that from 7 weeks post conception, expression of *CBE1* precedes that of *TEKT1*, but follows that of *FOXJ1* mRNA in developing human lung.

We also examined the expression of CBE1 protein by immunohistochemistry, using an anti-CBE1 polyclonal antibody. As differentiation of ciliated epithelium in the human airways occurs between 11 and 16 weeks post conception in the mid-late pseudoglandular stage (15;16), we used fetal lung tissues obtained at 10 and 12.3 weeks post conception. Immunoreactivity of the CBE1 protein was hardly detectable in 10 weeks post conception fetal airway tissue where no cilia were observed (fig. 5a,b). On the other hand, expression of CBE1 protein was strong in airway epithelium of lungs at 12.3 weeks post conception, when cilia were clearly visible (fig. 5c,d), consistent with a correlation of CBE1 expression and ciliogenesis. Positive signals were observed not only in columnar epithelial cells but also in basal epithelial cells of foetal lung (fig. 5c,d), in contrast to adult human bronchi (fig. 5e,f).

*Expression of CBE1 in embryonic/fetal lung tissue explant cultures*

Due to the difficulty in obtaining human fetal lungs after 11-12 weeks post conception, we cultured embryonic/fetal lung tissues *in vitro* in order to mimic *in vivo* development and assessed the induction of *CBE1* mRNA by RTqPCR. Prior to culture, expression of *CBE1* mRNA in embryonic lung taken 7 to 9 weeks post conception was scarcely detectable (CT values around 36-40) (data not shown), consistent with the semi-quantitative RT-PCR analyses (fig. 4a,b). However, when human fetal lungs at 9 weeks post conception were

cultured *in vitro*, *CBE1* mRNA was chronologically increased with a 10-fold increase in mRNA levels at day 12 (equivalent to 10.7 weeks post conception *in vivo*) ($P = 0.03$) and more than a 200-fold increase at day 18 (equivalent to 11.6 weeks post conception *in vivo*) ($P = 0.01$) compared to day 0 (fig. 6a). Expression of *FOXJ1* also showed a parallel increase of approximately 10-fold at day 12 ($P = 0.01$) and day 18 ($P = 0.01$), compared to day 0 (fig. 6b). The increase of expression levels was greater for *CBE1* than for *FOXJ1* mRNA, probably because *FOXJ1* was already significantly expressed before the start of culture at 9 weeks post conception (fig. 4a). On the other hand, we were unable to detect expression of *TEKT1* mRNA during this *ex vivo* differentiation even after 18 days in culture (data not shown), confirming that expression of *TEKT1* mRNA is absent when both *CBE1* and *FOXJ1* mRNAs are significantly expressed, as observed in the *in vivo* analyses (fig. 4a,b). Protein expression of CBE1 in the cultured human fetal lungs was also investigated by immunohistochemistry, showing no staining at day 0 (9 weeks post conception) and day 6, but substantial staining in the developing epithelium at day 18 (equivalent to 11.6 weeks post conception *in vivo)*, when ciliary structures were visible (fig. 6c). Figure 7 shows a schematic representation summarizing the temporal pattern of *Cbe1/CBE1* expression in developing mouse and human lungs. Although we were limited to obtaining human lung samples during the pseudoglandular stage of development, there was good concordance between the *Cbe1/CBE1* expression profiles in both species.

## DISCUSSION

In the healthy airways, ciliated cells represent more than 80% of the total columnar epithelial cell population, and are interspersed with mucus-secreting goblet cells. However, in chronic airway diseases such as asthma, cystic fibrosis, and chronic bronchitis, the number of goblet cells markedly increases (17;18). This results in accelerated production and/or secretion of mucus, which in turn causes resistance to air flow, and abrogates normal mucociliary function. The consequences of these changes include increased sputum production, airway narrowing and disease exacerbation, or asphyxiation in the case of fatal asthma attacks (19). Therefore, efficient and appropriate repair in bronchial epithelium, leading to enrichment of ciliated cells, would be of great benefit in airway diseases. However, the cellular and molecular mechanisms of ciliogenesis have not been fully investigated.

The factors required for the commitment of an undifferentiated airway epithelial cell to a ciliated cell are not fully known. As already indicated, studies using *Foxj1* null mice, which fail to develop motile cilia, clearly show that *Foxj1* plays a crucial role for ciliogenesis (3;4). However, it is important to note that in *Foxj1*$^{-/-}$ mice, cilia precursors are present inside airway epithelial cells but fail to dock at the apical membrane and form cilia (4). Gain of function analyses using an adenovirus (or lentivirus) expression vector and ALI-cultured mouse tracheal epithelial cells has shown that *Foxj1* alone is not sufficient to induce a program of ciliogenesis (5). Indeed, *in*

*vivo* and *in vitro* studies show that *Foxj1* functions in the late stages of ciliogenesis to regulate basal body docking and axoneme formation in cells previously committed to the ciliated cell phenotype (20;21).

In order to study expression of Cbe1/CBE1 during lung development, we used an *in vivo* approach with murine lungs and an *ex vivo* approach using human embryonic lung tissue explants. The explant culture provided a useful model for studying early human lung development and during the period of the experiments (up to 18 days) we observed maintenance of branching morphogenesis. In terms of CBE1 protein expression, induction of expression after 18 days *in vitro* (9 weeks post conception + 2.6 weeks ex vivo) was similar to that observed *in vivo* at 12 weeks suggesting that this aspect of cellular programming was normal during the culture period. Although growth of the tissue eventually becomes limited by the requirement for a blood supply, this model offers the potential for studying molecular events that control the pseudoglandualr stage of development..

In our previous study, we observed that transcription of *CBE1*, *FOXJ1* and TEKT*1* mRNAs was synchronous during *in vitro* differentiation using an air-liquid interface (ALI) culture; all of these ciliated cell-associated genes were switched on at 14 days after the start of ALI, when RNA was extracted at day 7, 14 and 21 (6). However, in murine lungs *in vivo* and using human

fetal lungs *ex vivo*, we have observed a distinct order of expression during differentiation of ciliated cells in the airway epithelium where *Foxj1*/*FOXJ1* was earlier than *Cbe1*/*CBE1* mRNAs, while expression of *TEKT1* was undetectable up to 11-12 weeks post conception. A further difference between the human adult and embryonic tissue was the protein distribution of CBE1. In adult bronchial epithelium, CBE1 protein immunostaining was restricted to the columnar epithelial cells, whereas in the embryonic lung tissue it could be detected in basal, as well as columnar, epithelial cells from 12 weeks post conception. Whether these basal cells represent early ciliated cell progenitors within the pseudostratified epithelium remains to be determined. Their absence in adult bronchial epithelium may reflect slower cell turnover as compared with the much more rapidly growing embryonic airways. Alternatively, these findings may suggest that the *in vitro* differentiation system using adult cells does not necessarily reflect fetal lung differentiation, even though it produces fully differentiated columnar epithelial cells possessing beating cilia and mucous-secreting goblet cells (22).

Table 1 shows a comparison of the stages of mouse and human lung development according to histological criteria (23-26). Chronological expression of *Foxj1* and *Cbe1* mRNA in developing mouse lungs was consistent with that observed in human fetal airways, in that induction of *Foxj1* was earlier (E15) than that of *Cbe1* (E16) in late pseudoglandular stage of development (fig.3ab & 7). The result obtained for *Foxj1* expression is also consistent with a

previous report showing that *Foxj1* mRNA was detectable from E14.5 in embryonic lungs by Northern blot and in situ hybridization analyses (27). However, it should be noted that we observed a biphasic expression of *Cbe1* mRNA with significantly higher expression of *Cbe1* at E11, during the formation of lung buds, when the expression of *Foxj1* was absent (fig. 3ab & 7). This suggests that *Cbe1* may function during the early *and* later stages of lung development E11 in mice corresponds to 4-5 weeks post conception in human embryos (Table 1) and is a time when expression of *FOXJ1* mRNA is absent (28). Unfortunately, we were unable to confirm *CBE1* mRNA expression in human lungs as we could not obtain appropriate embryonic tissues due to ethical reasons. Thus, whether human embryos at this early stage also transiently express *CBE1* mRNA remains to be determined (fig. 7).

It was unexpected to find significant expression of *Tekt1* mRNA from E11 onwards, because we detected no *TEKT1* mRNA in human fetal lungs at 10 weeks post conception, when both *CBE1* and *FOXJ1* mRNAs were observed. However, this chronological pattern of expression may be consistent with a previous study reporting by Northern blotting that *Tekt1* mRNA was detectable from ED12 onwards (29). These data suggest that the regulatory mechanisms controlling transcription of TEKT1/*Tekt1* mRNAs during embryogenesis are different between human and mice, although these orthologs are structurally highly conserved (82% identical in amino acid sequences; (30).

*Foxa1* and *Foxa2*, structurally homologous transcription factors, are now known to play a crucial role in the differentiation of bronchial epithelium. Conditional disruption of *Foxa1* and *Foxa2* reduced the expression of several marker genes in lung epithelium including surfactant protein, Clara cell secretory protein and *Foxj1* (12), suggesting that these transcription factors positively regulate expression of *Foxj1* mRNA directly or indirectly. Protein expression of Foxa1 and Foxa2 in the mouse embryo has been precisely evaluated by immunohistochemistry, showing that both transcription factors can be detected in the nuclei in the lung bud at E10.5, and in the lung epithelium thereafter (13). This is consistent with our present study showing that *Foxa1* and *Foxa2* mRNAs were constantly detectable in the mouse embryonic lungs at E11 and thereafter (fig. 3c,d). We have previously reported that forced expression of *FOXJ1* cDNA in a human bronchial epithelial cell line 16HBE 14o(-) induced endogenous *TEKT1* mRNA expression but not *CBE1*, indicating that *FOXJ1* alone is not sufficient for the transcription of *CBE1* (6). The presence of the *Cbe1* mRNA at E11 may suggest that *Cbe1* is induced by other transcription factor(s) such as Foxa1 or Foxa2 that function upstream of Foxj1. Chronological expression patterns during later stages of lung development may also suggest that Cbe1 cooperates with Foxj1 to control mucociliary differentiation. Further functional studies are now required to define the role of Cbe1/CBE1 in lung epithelial differentiation and ciliogenesis.

**Acknowledgements**

The authors thank Dr. R. M. Powell for designing and evaluating primers and probes for qPCR. The authors also thank generous support from the Asthma, Allergy and Inflammation Research (AAIR) and Hope Charities. STH is an MRC Clinical Professor.

## FIGURE LEGENDS

**FIGURE 1.** Characterization of the *Cbe1* cDNA. (a) Nucleotide and deduced amino acid sequences of the lung-expressed full-length *Cbe1* cDNA. There is a 5 bp-insertion in one of the splicing sites within the cDNA, leading to an alternative ORF with a longer and different carboxyl terminus (ORF2, shown in bold). GenBank accession number: DQ873295 (ORF1), DQ873296 (ORF2). Two arginine residues which were shown to be responsible for its nuclear localization for CBE1 are boxed. (b) Schematic view of the intron/exon organization, comparison of the amino acid chain length of splicing variants of *Cbe1* mRNA and analysis of their expression in lung and testis. Unshaded boxes represent untranslated regions but the 5'-untranslated sequences of the long and short forms are different to each other due to use of an alternate promoter. Semi-quantitative RT-PCR was carried out using variant-specific primers. PCR cycles were 35 for each *Cbe1* mRNA variants. (c) Comparison of the amino acid sequences of Cbe1 and CBE1. Identical residues are shown by asterisks.

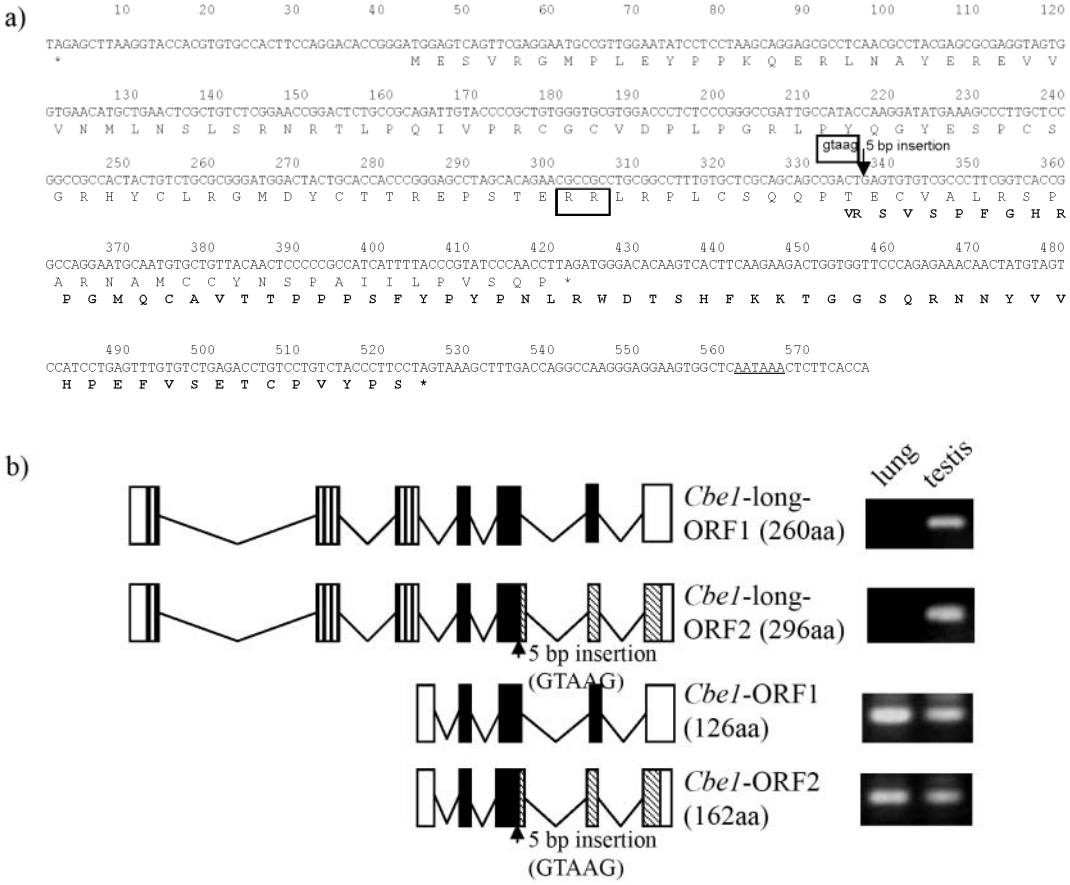


FIGURE 1.

**FIGURE 2.** (a) Tissue distribution of *Cbe1* mRNA analysed by semi-quantitative RT-PCR. Total RNA samples were isolated from indicated different organs of adult mouse, followed by cDNA synthesis and PCR using primers detecting long or short forms (common to ORF1 and

ORF2) of *Cbe1*. PCR cycles were 35 for *Cbe1* cDNA and 25 for *G3pdh cDNA*. RT-PCR using variant (ORF1, ORF2)-specific primers resulted in a similar pattern of distribution (data not shown). (b) Reactivity of anti-CBE1 antibodies against recombinant CBE1 and Cbe1 proteins. Expression plasmids encoding full-length ORF1 or ORF2 of *CBE1* or *Cbe1* cDNAs, or vector alone (pcDNA3.1) were transiently introduced into HEK293 cells by lipofection. After 48h, cellular extracts were separated in 15% SDS-PAGE, followed by immunoblotting using anti-CBE1 antiserum. Bands of ORF1 and ORF2 are indicated by arrows.

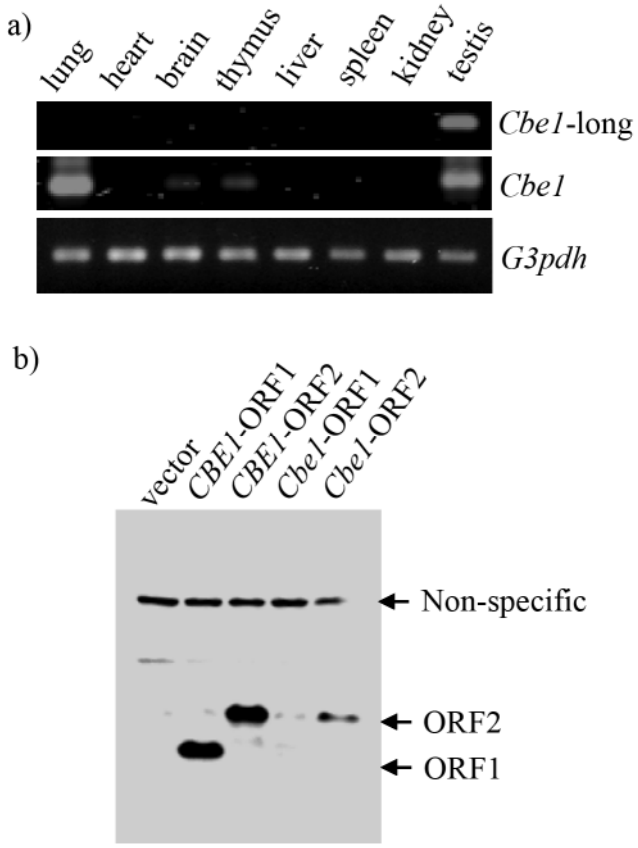


FIGURE 2.

**FIGURE 3.** Chronological expression of *Foxj1* (a), *Cbe1* (b), *Foxa1* (c), *Foxa2* (d) and *Tekt1* (e) mRNAs during all stages of mouse lung development (f). Mouse lung tissues were dissected from embryos at indicated days after gestation (E, embryonic day), new-born mice at indicated days of post partum (P), and adult mice (AM). Total RNA was isolated and cDNA was synthesized for SYBR green qPCR analyses. The expression level is given relative to the level of *Gapdh* mRNA , which was used as housekeeping gene. Data are from 5-8 lungs per group.

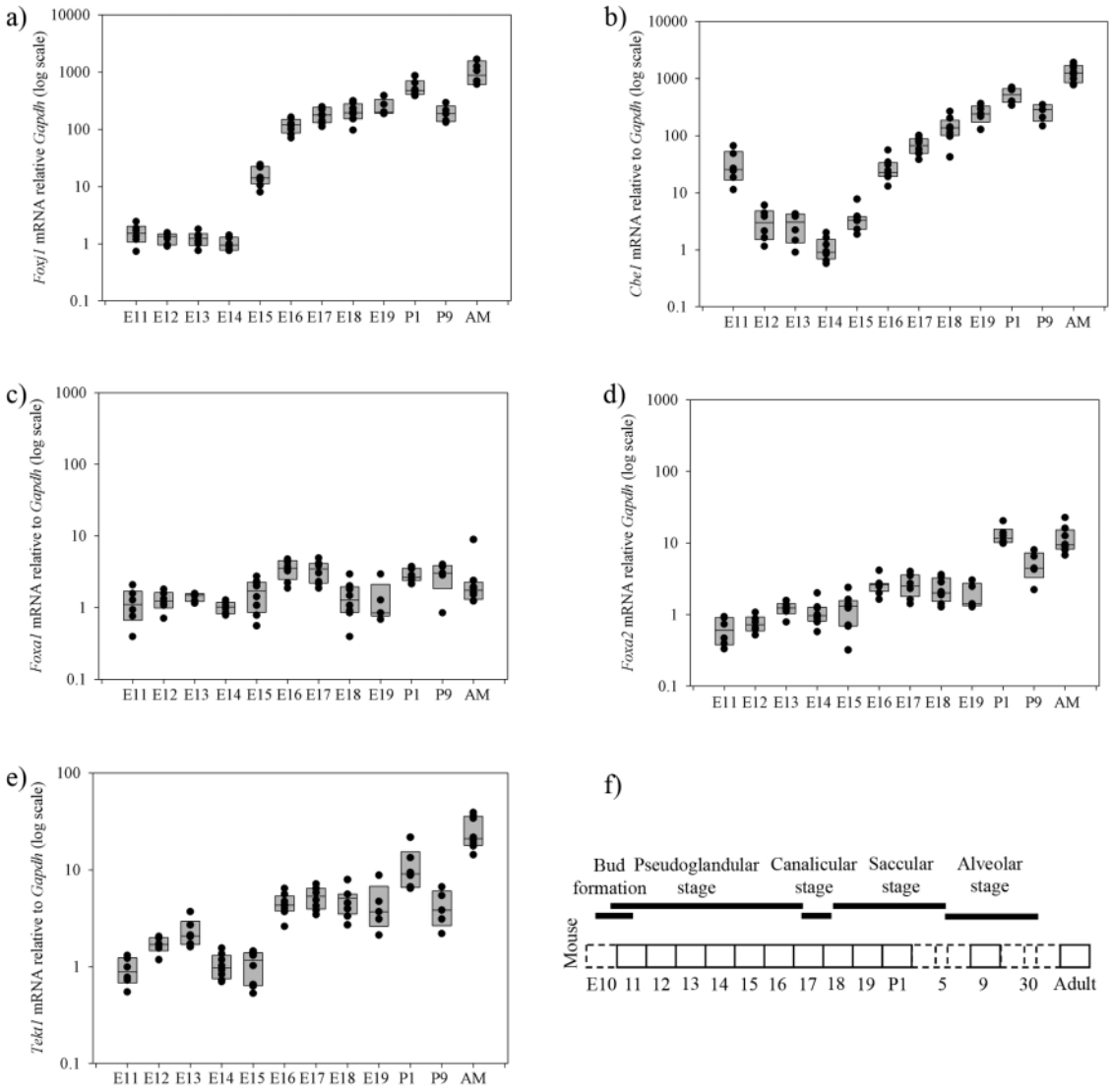


FIGURE 3 revised.

**FIGURE 4.** Expression of *CBE1* mRNA in human embryonic/fetal lungs. (a) Detection of mRNA analysed by semi-quantitative RT-PCR. Bronchial tissues were taken from human embryos/fetuses at the indicated stages. Each lane represents different donors. PCR cycles were 35 for analysis of *FOXJ1*, *CBE1* and *TEKT1* and 25 for *G3PDH* cDNAs. cDNA from ALI-differentiated bronchial epithelial cells (at day 14) or that from cultured bronchial

fibroblasts was used as positive or negative control, respectively. (b) Nested PCR (15 cycles) was carried out for *CBE1* and *TEKT1* mRNAs using diluted reaction products (1:10) of the standard RT-PCR analyses (panel A) and the respective nested primers.

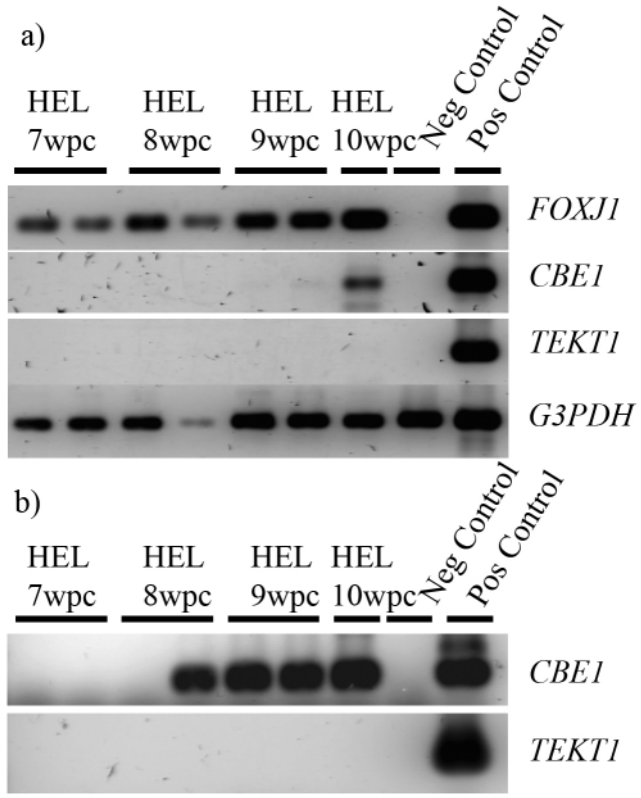


FIGURE 4 revised.

**FIGURE 5.** (a-f) Expression of the CBE1 protein analysed by immunohistochemistry. Brown immunostaining using diaminobenzidine as chromagen showing the presence of CBE1 which is not detectable at 10 weeks post conception (a,b), but is strong in epithelium at 12.3 weeks post conception (c,d) where ciliary structures are visible (arrowheads); CBE1 immunostaining was detected not only in columnar cells but also basal cells. In contrast, staining is confined to columnar epithelial cells in adult human bronchi (e,f). Scale bars = 60 μm (a,c,e), or 20 μm (b,d,f).

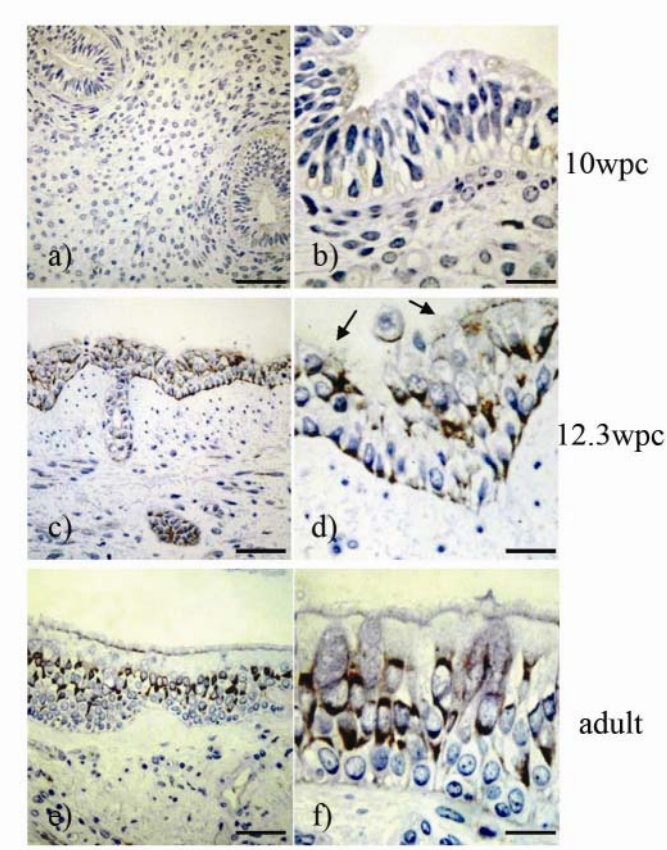

FIGURE 5.

**FIGURE 6.** Chronological expression of *CBE1* (a) and *FOXJ1* (b) mRNAs during *ex vivo* differentiation of human embryonic/fetal lungs. mRNA expression was analysed by RT-qPCR. Isolated human fetal lungs (7- 9 weeks post conception) were cultured and total RNA isolated at the indicated days after the start of culture. Each dot represents the result obtained from independent samples. Data are normalized relative to the house keeping gene, *beta-actin* (*ACTB*) mRNA levels. (c) Protein expression was analysed by immunohistochemistry. Cultured (day 6 or 18) or non-cultured (day 0) embryonic lung tissues were fixed and stained

as in fig. 5, except that 3-amino-9-ethylcarbazole was used as chromagen to give positive red immunostaining. Scale bar = 100 μm.

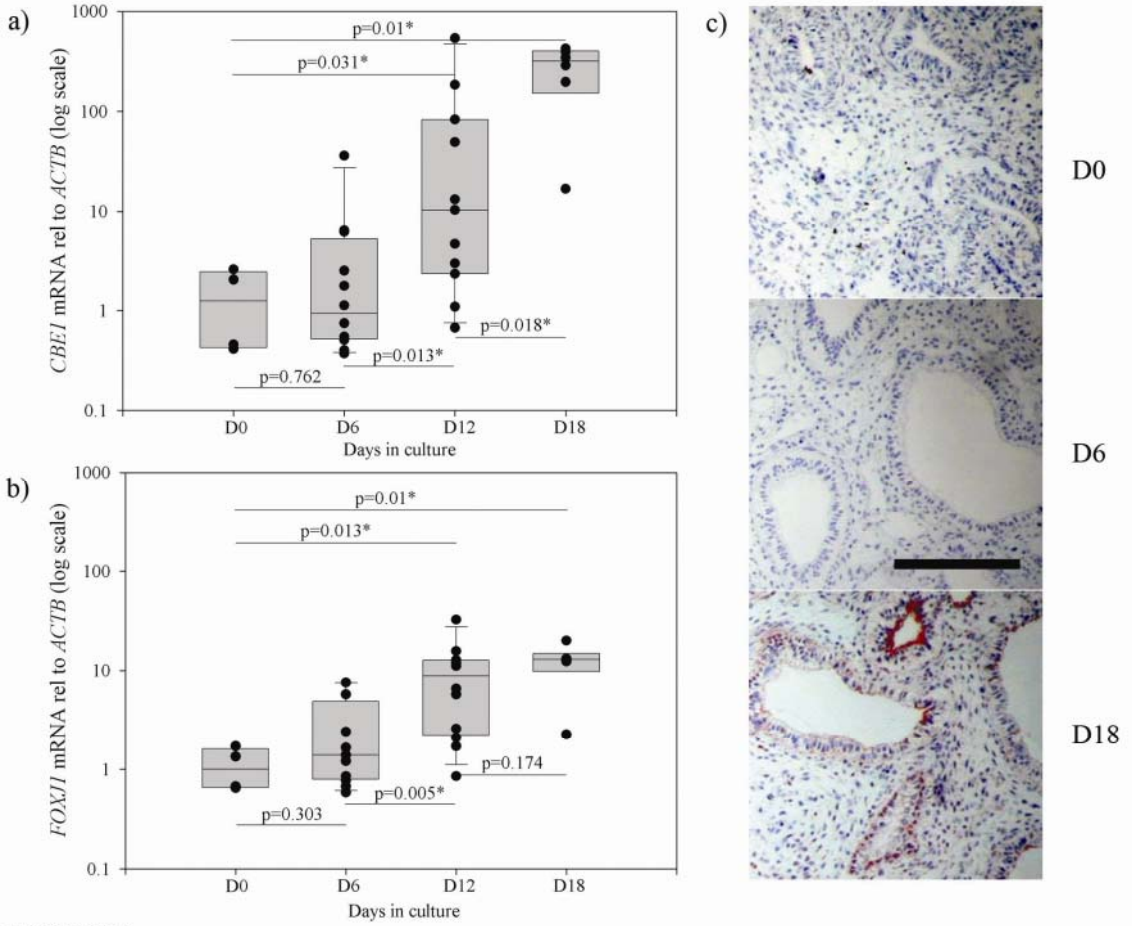


FIGURE 6.

**FIGURE 7.** Schematic representation of stages of lung development and the temporal pattern of *Cbe1* (gray scales) and *CBE1* (diagonal lined) mRNA expression in mouse and human lungs and CBE1 protein (horizontal dashed) expression in human lungs. Embryonic day (E), post partum day (P), weeks post conception (W), year (Y).

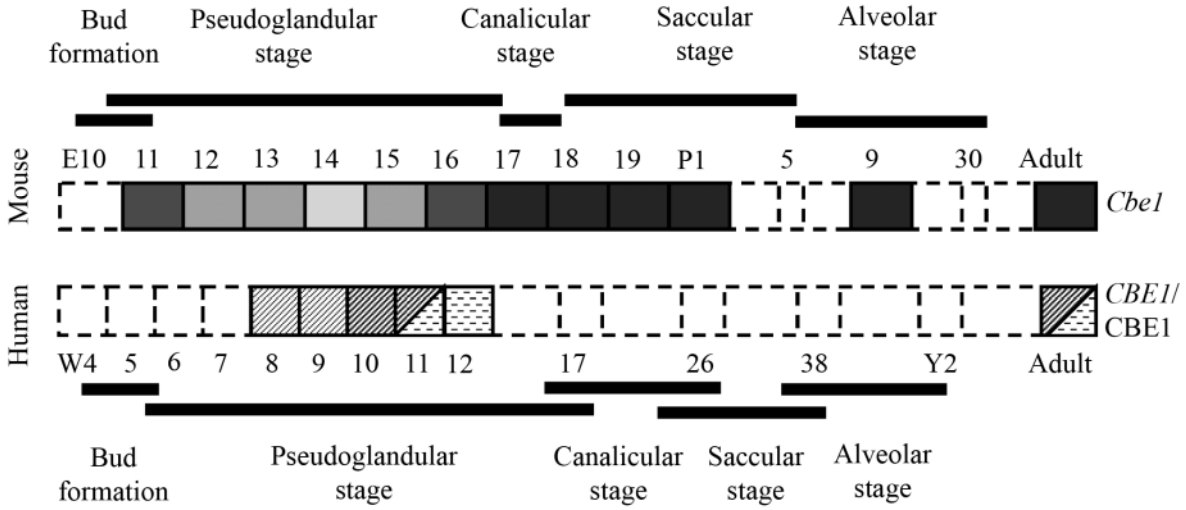


FIGURE 7 revision

**TABLE 1.** Stages of lung development, adapted from References (23-26)

| Stages of mouse and human lung development according to histological criteria | | |
|---|---|---|
| Stage | Mouse<br>(days post conception) | Human<br>(weeks post conception) |
| Bud formation start | ~9.5 | 4 (~26 days post conception) |
| Pseudoglandular | 9.5 – 16.6 | 5 – 17 |
| Canalicular | 16.6 – 17.4 | 16 – 26 |
| Saccular | 17.4 – 5days after birth | 24 – 38 |
| Alveolar | Day 5 – day 30 after birth | 36 – 1 to 2 years after birth |

References

(1) Mills PR, Davies RJ, Devalia JL. Airway epithelial cells, cytokines, and pollutants. Am J Respir Crit Care Med 1999 Nov;160(5 Pt 2):S38-S43.

(2) Whitsett JA. Intrinsic and innate defenses in the lung: intersection of pathways regulating lung morphogenesis, host defense, and repair. J Clin Invest 2002 Mar;109(5):565-9.

(3) Chen J, Knowles HJ, Hebert JL, Hackett BP. Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. J Clin Invest 1998 Sep 15;102(6):1077-82.

(4) Brody SL, Yan XH, Wuerffel MK, Song SK, Shapiro SD. Ciliogenesis and left-right axis defects in forkhead factor HFH-4-null mice. Am J Respir Cell Mol Biol 2000 Jul;23(1):45-51.

(5) You Y, Huang T, Richer EJ, Schmidt JE, Zabner J, Borok Z, et al. Role of f-box factor foxj1 in differentiation of ciliated airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 2004 Apr;286(4):L650-L657.

(6) Yoshisue H, Puddicombe SM, Wilson SJ, Haitchi HM, Powell RM, Wilson DI, et al. Characterization of ciliated bronchial epithelium 1, a ciliated cell-associated gene induced during mucociliary differentiation. Am J Respir Cell Mol Biol 2004 Jul 8;31((5)):491-500.

(7) Haitchi HM, Powell RM, Shaw TJ, Howarth PH, Wilson SJ, Wilson DI, et al. ADAM33 Expression in Asthmatic Airways and Human Embryonic Lungs. Am J Respir Crit Care Med 2005 May 1;171(9):958-65.

(8) Britten KM, Howarth PH, Roche WR. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. Biotech Histochem 1993 Sep;68(5):271-80.

(9) Powell RM, Wicks J, Holloway JW, Holgate ST, Davies DE. The splicing and fate of ADAM33 transcripts in primary human airways fibroblasts. Am J Respir Cell Mol Biol 2004 Jul;31(1):13-21.

(10) Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 1987 Oct 26;15(20):8125-48.

(11) Wan H, Kaestner KH, Ang SL, Ikegami M, Finkelman FD, Stahlman MT, et al. Foxa2 regulates alveolarization and goblet cell hyperplasia. Development 2004 Feb;131(4):953-64.

(12) Wan H, Dingle S, Xu Y, Besnard V, Kaestner KH, Ang SL, et al. Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. J Biol Chem 2005 Apr 8;280(14):13809-16.

(13) Besnard V, Wert SE, Hull WM, Whitsett JA. Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. Gene Expr Patterns 2004 Dec;5(2):193-208.

(14) Iguchi N, Tanaka H, Nakamura Y, Nozaki M, Fujiwara T, Nishimune Y. Cloning and characterization of the human tektin-t gene. Mol Hum Reprod 2002 Jun;8(6):525-30.

(15) Gaillard DA, Lallement AV, Petit AF, Puchelle ES. In vivo ciliogenesis in human fetal tracheal epithelium. Am J Anat 1989 Aug;185(4):415-28.

(16) Jeffery P.K. The development of large and small airways. Am J Resp Crit Care Med 1998;157:S174-S180.

(17) Rose MC, Nickola TJ, Voynow JA. Airway mucus obstruction: mucin glycoproteins, MUC gene regulation and goblet cell hyperplasia. Am J Respir Cell Mol Biol 2001 Nov;25(5):533-7.

(18) Fahy JV. Goblet cell and mucin gene abnormalities in asthma. Chest 2002 Dec;122(6 Suppl):320S-6S.

(19) Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. Chest 1992 Apr;101(4):916-21.

(20) Huang T, You Y, Spoor MS, Richer EJ, Kudva VV, Paige RC, et al. Foxj1 is required for apical localization of ezrin in airway epithelial cells. J Cell Sci 2003 Dec 15;116(Pt 24):4935-45.

(21) Gomperts BN, Gong-Cooper X, Hackett BP. Foxj1 regulates basal body anchoring to the cytoskeleton of ciliated pulmonary epithelial cells. J Cell Sci 2004 Mar 15;117(Pt 8):1329-37.

(22) Puddicombe SM, Page A, Swallow DM, Thornton DJ, Holgate ST, Davies DE. Characterisation of the mucosecretory phenotype induced by chronic exposure to

interleukin (IL)-13 *in vitro*. Am.J.Respir.Crit Care Med. 167, A454. 2003.

(23) Strachan T, Lindsay S, Wilson DI. Molecular genetics of early human development. Oxford: BIOS Scientific Publishers Ltd; 1997.

(24) Rosenthal M, Bush A. The growing lung: normal development, and the long-term effects of pre- and postnatal insults . Eur Respir Mon 2002;19:1-24.

(25) Perl AK, Whitsett JA. Molecular mechanisms controlling lung morphogenesis. Clin Genet 1999 Jul;56(1):14-27.

(26) Ten Have-Opbroek AA. Lung development in the mouse embryo. Exp Lung Res 1991 Mar;17(2):111-30.

(27) Hackett BP, Brody SL, Liang M, Zeitz ID, Bruns LA, Gitlin JD. Primary structure of hepatocyte nuclear factor/forkhead homologue 4 and characterization of gene expression in the developing respiratory and reproductive epithelium. Proc Natl Acad Sci U S A 1995 May 9;92(10):4249-53.

(28) Pelletier GJ, Brody SL, Liapis H, White RA, Hackett BP. A human forkhead/winged-helix transcription factor expressed in developing pulmonary and renal epithelium. Am J Physiol 1998 Mar;274(3 Pt 1):L351-L359.

(29) Norrander J, Larsson M, Stahl S, Hoog C, Linck R. Expression of ciliary tektins in brain and sensory development. J Neurosci 1998 Nov 1;18(21):8912-8.

(30) Xu M, Zhou Z, Cheng C, Zhao W, Tang R, Huang Y, et al. Cloning and characterization of a novel human TEKTIN1 gene. Int J Biochem Cell Biol 2001 Dec;33(12):1172-82.