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Chronological expression of *Ciliated Bronchial Epithelium 1* during pulmonary development

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ABSTRACT: ***Ciliated Bronchial Epithelium* (*CBE*) *1* is a novel gene, which is expressed in ciliated cells. As cilia are important during embryogenesis, the present authors characterised 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.**

The 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 (E)16, 1 day later than *Foxj1*, which is 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: ***Ciliated bronchial epithelium 1*, ciliogenesis, embryonic/fetal lung development, epithelium, forkhead box factor *J1***

Cilia are finger-like appendages that are microtubule (MT)-based organelles. They are classified according to their MT components as 9+2 (motile) and 9+0 (primary) cilia. The 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) 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, which have yet to be characterised.

Recently, a novel gene was characterised, *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.

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Expression studies showed that CBE1 is localised to the nuclear or perinuclear regions of cells, implying that CBE1 might be a nucleocytoplasmic shuttling protein, although no clear function for CBE1 has been described as yet. Although strong induction of the *CBE1* mRNA during *in vitro* mucociliary differentiation of primary bronchial epithelial cells has been shown, its expression during lung development has not been investigated. The present authors 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 (HEL) explants cultured *in vitro*.

MATERIALS AND METHODS

Cloning and characterisation 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, USA) 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 supplementary data.

Isolation of mouse lungs

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

Isolation of human fetal lungs and ex vivo *differentiation*

Human fetal lung tissues were collected from females 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 $\leqslant$18 days.

Immunohistochemistry

Human fetal lungs were processed into glycol methacrylate resin and 2-μm sections were 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 $\mu g \cdot mL^{-1}$.

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 synthesised as previously described [6]. For semi-quantitative PCR and nested PCR, cDNA was amplified using specific primers as described in the online supplementary data. PCR products were separated in 1.5% agarose gel and visualised with ethidium bromide or Vistra Green (Amersham Biosciences, Amersham, UK). RT-quantitative PCR (RT-qPCR) was performed using an IcyclerIQ system (Bio-Rad, Hemel Hempstead, UK) [9]. Relative expression levels were calculated using the ΔΔCt (threshold cycle) method. Specific primers, probes and experimental conditions are provided in the online supplementary data. The results were expressed relative to either *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) or *beta-actin* (*ACTB*) mRNA levels, which were used as housekeeping genes.

Statistical analyses

Statistical analysis was undertaken using the Mann–Whitney test. A p-value <0.05 was considered as significant.

RESULTS

Characterisation of Cbe1 *and its tissue distribution*

To compare the expression profile of *CBE1* mRNA with that of its rodent counterpart, the present authors cloned and characterised the murine ortholog of *CBE1* (*Cbe1*). A 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 a small ORF consisting of 126 amino acids was found. 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 that were sequence analysed showed a 5-bp insertion at one of the splicing sites, resulting in a frame shift. In turn, this generated 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 that could serve as one of the nuclear localisation signals in CBE1 [6] are conserved.

The present authors 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 analysed tissues (heart, liver, spleen or kidney; fig. 2a), This latter finding is in contrast with *CBE1* mRNA which is also expressed in heart and kidney [7], although *Cbe1* mRNA could be detected 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*.

a)

```
        10        20        30        40        50        60        70        80        90       100       110       120
TAGAGCTTAAGGTACCACGTGTGCCACTTCCAGGACACCGGGATGGAGTCAGTTCGAGGAATGCCGTTGGAATATCCTCCTAAGCAGGAGCGCCTCAACGCCTACGAGCGCGAGGTAGTG
 *                                    M  E  S  V  R  G  M  P  L  E  Y  P  P  K  Q  E  R  L  N  A  Y  E  R  E  V  V

       130       140       150       160       170       180       190       200       210       220       230       240
GTGAACATGCTGAACTCGCTGTCTCGGAACCGGACTCTGCCGCAGATTGTACCCCGCTGTGGGTGCGTGGACCCTCTCCCGGGCCGATTGCCATACCAAGGATATGAAAGCCCTTGCTCC
 V  N  M  L  N  S  L  S  R  N  R  T  L  P  Q  I  V  P  R  C  G  C  V  D  P  L  P  G  R  L  P  Y  Q  G  Y  E  S  P  C  S
                                                                                                GTAAG 5 bp insertion
       250       260       270       280       290       300       310       320       330       340       350       360
GGCCGCCACTACTGTCTGCGCGGGATGGACTACTGCACCACCCGGGAGCCTAGCACAGAACGCCGCCTGCGGCCTTTGTGCTCGCAGCAGCCGACTGAGTGTGTCGCCCTTCGGTCACCG
 G  R  H  Y  C  L  R  G  M  D  Y  C  T  T  R  E  P  S  T  E  R  R  L  R  P  L  C  S  Q  Q  P  T  E  C  V  A  L  R  S  P
                                                                                                VR  S  V  S  P  F  G  H  R

       370       380       390       400       410       420       430       440       450       460       470       480
GCCAGGAATGCAATGTGCTGTTACAACTCCCCCGCCATCATTTTACCCGTATCCCAACCTTAGATGGGACACAAGTCACTTCAAGAAGACTGGTGGTTCCCAGAGAAACAACTATGTAGT
 A  R  N  A  M  C  C  Y  N  S  P  A  I  I  L  P  V  S  Q  P  *
 P  G  M  Q  C  A  V  T  T  P  P  P  S  F  Y  P  Y  P  N  L  R  W  D  T  S  H  F  K  K  T  G  G  S  Q  R  N  N  Y  V  V

       490       500       510       520       530       540       550       560       570
CCATCCTGAGTTTGTGTCTGAGACCTGTCCTGTCTACCCTTCCTAGTAAAGCTTTGACCAGGCCAAGGGAGGAAGTGGCTCAATAAACTCTTCACCA
 H  P  E  F  V  S  E  T  C  P  V  Y  P  S  *
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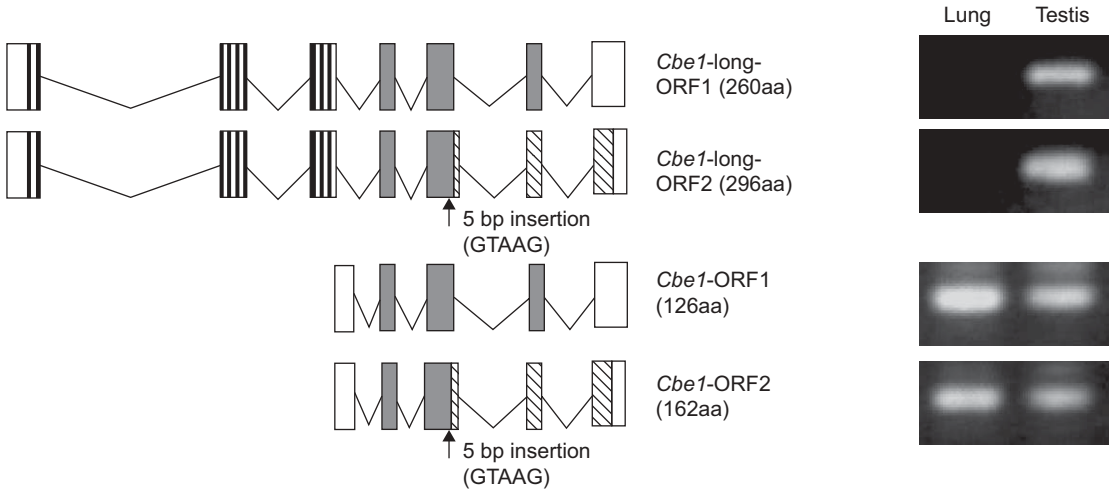
b)



c)

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human CBE1 ORF1    1:METAVRGMPLECPPRPERLNAYEREVMVNMLNSLSRNQQLPRITPRCGCVDPLPGRLPFH 60
mouse CBE1 ORF1    1:MESV-RGMPLEYPPKQERLNAYEREVVVNMLNSLSRNRTLPQIVPRCGCVDPLPGRLPYQ 59
                       **   ****** **  ********** **********  ** * **************

human CBE1 ORF1   61:GYESACSGRHYCLRGMDYYASGAPCTDRRLRPWCREQPTMCTSLRAPARNAVCCYNSPAV 120
mouse CBE1 ORF1   60:GYESPCSGRHYCLRGMDYCTTREPSTERRLRPLCSQQPTECVALRSPARNAMCCYNSPAI 119
                      **** **************     * * ***** *  *** *  ** ***** *******

human CBE1 ORF1  121:ILPISEP                                                    127
mouse CBE1 ORF1  120:ILPVSQP                                                    126
                     *** * *

human CBE1 ORF2    1:METAVRGMPLECPPRPERLNAYEREVMVNMLNSLSRNQQLPRITPRCGCVDPLPGRLPFH 60
mouse CBE1 ORF2    1:MESV-RGMPLEYPPKQERLNAYEREVVVNMLNSLSRNRTLPQIVPRCGCVDPLPGRLPYQ 59
                       **   ****** **  ********** **********  ** * **************

human CBE1 ORF2   61:GYESACSGRHYCLRGMDYYASGAPCTDRRLRPWCREQPTVRCVPPYEHRPGMQCAVTTPP 120
mouse CBE1 ORF2   60:GYESPCSGRHYCLRGMDYCTTREPSTERRLRPLCSQQPTVRSVSPFGHRPGMQCAVTTPP 119
                      **** **************     * * ***** *  ***** * *  **************

human CBE1 ORF2  121:PSYYPYPNLRWDTSHFKKSGGPQRNNYVIHPEFVSETYPDYRCW                164
mouse CBE1 ORF2  120:PSFYPYPNLRWDTSHFKKTGGSQRNNYVVHPEFVSETCPVYPS-                162
                     ** *************** ** ****** ******** * *
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FIGURE 1. Characterisation 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 open reading frame (ORF) with a longer and different carboxyl terminus (ORF2; GTAAG). Two arginine residues which were shown to be responsible for its nuclear localisation for CBE1 are shown. GenBank accession number: DQ873295 (ORF1), DQ873296 (ORF2). b) Schematic view of the intron/exon organisation, comparison of the amino acid chain length of splicing variants of *Cbe1* mRNA and analysis of their expression in lung and testis. The open 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.

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 with recombinant CBE1 when analysed by Western blotting (fig. 2b). Therefore, the present authors chose to use RT-qPCR analyses to investigate the chronological expression of *Cbe1* mRNA and other ciliogenesis-related genes during embryogenesis.

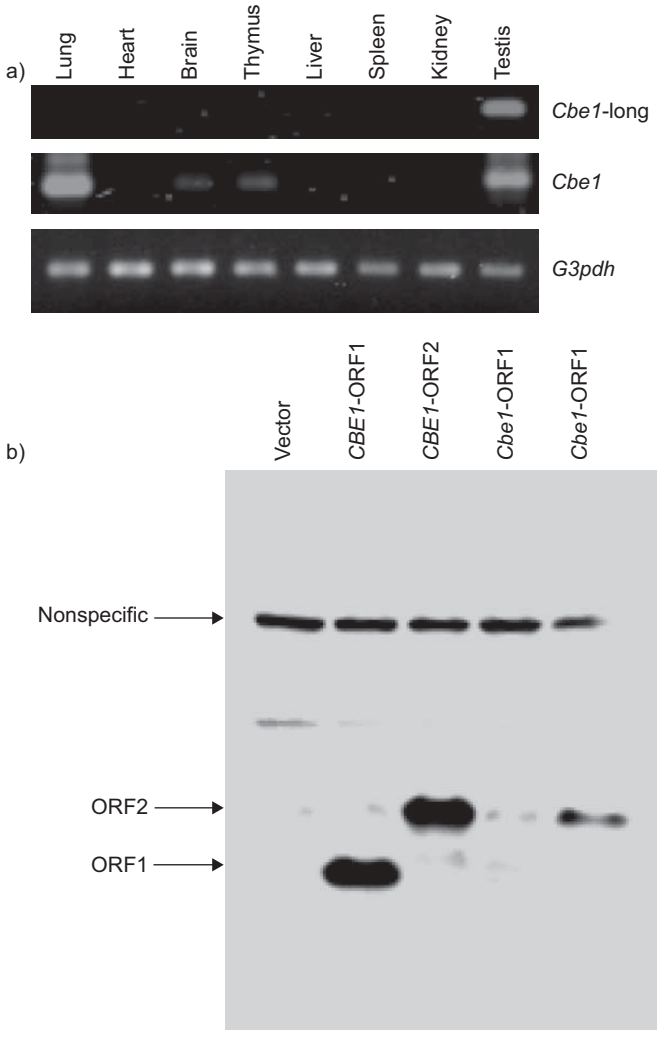

FIGURE 2. a) Tissue distribution of *Cbe1* mRNA analysed by semi-quantitative RT-PCR. Total RNA samples were isolated from indicated different organs of adult mouse, followed by cDNA synthesis and PCR using primers detecting long or short forms (common to open reading frame (ORF)1 and ORF2) of *Cbe1*. 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 48 h, cellular extracts were separated in 15% SDS-PAGE, followed by immunoblotting using anti-CBE1 antiserum. Bands of ORF1 and ORF2 are indicated by the arrows.

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 with the basal level at E14. *Foxj1* mRNA chronologically increased thereafter, up to 342±71-fold in AM. Expression of *Cbe1* mRNA increased 15.4±7.4-fold at E16 compared with the basal level at E14, which was later than *Foxj1*, and increased 447±99-fold in AM (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 three to five-fold (*Foxa2*; fig. 3d) from E11 to adult. This is consistent with a previous report which stated 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 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 with E12–E14; however, this was not observed for *Foxj1* (fig. 3a and b), suggesting that Cbe1 has a distinct function in early lung development.

Expression of CBE1 *in HEL*

The expression of *CBE1* mRNA in HEL was analysed using semi-quantitative RT-PCR, which showed a low but detectable amount of *CBE1* mRNA at 10 weeks post-conception. In contrast, *FOXJ1* mRNA was consistently observed from 7–10 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, the present authors performed nested PCR which revealed that *CBE1* mRNA was already present at 8–9 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.

The expression of CBE1 protein by immunohistochemistry was also examined using an anti-CBE1 polyclonal antibody. As differentiation of ciliated epithelium in the human airways occurs between 11–16 weeks post-conception in the mid-late pseudoglandular stage [15, 16], the present authors 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 and b). However, expression of CBE1 protein was strong in airway epithelium of lungs at 12.3 weeks post-conception when cilia were clearly visible (fig. 5c and d), and 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 fetal lung (fig. 5c and d), in contrast to adult human bronchi (fig. 5e and f).

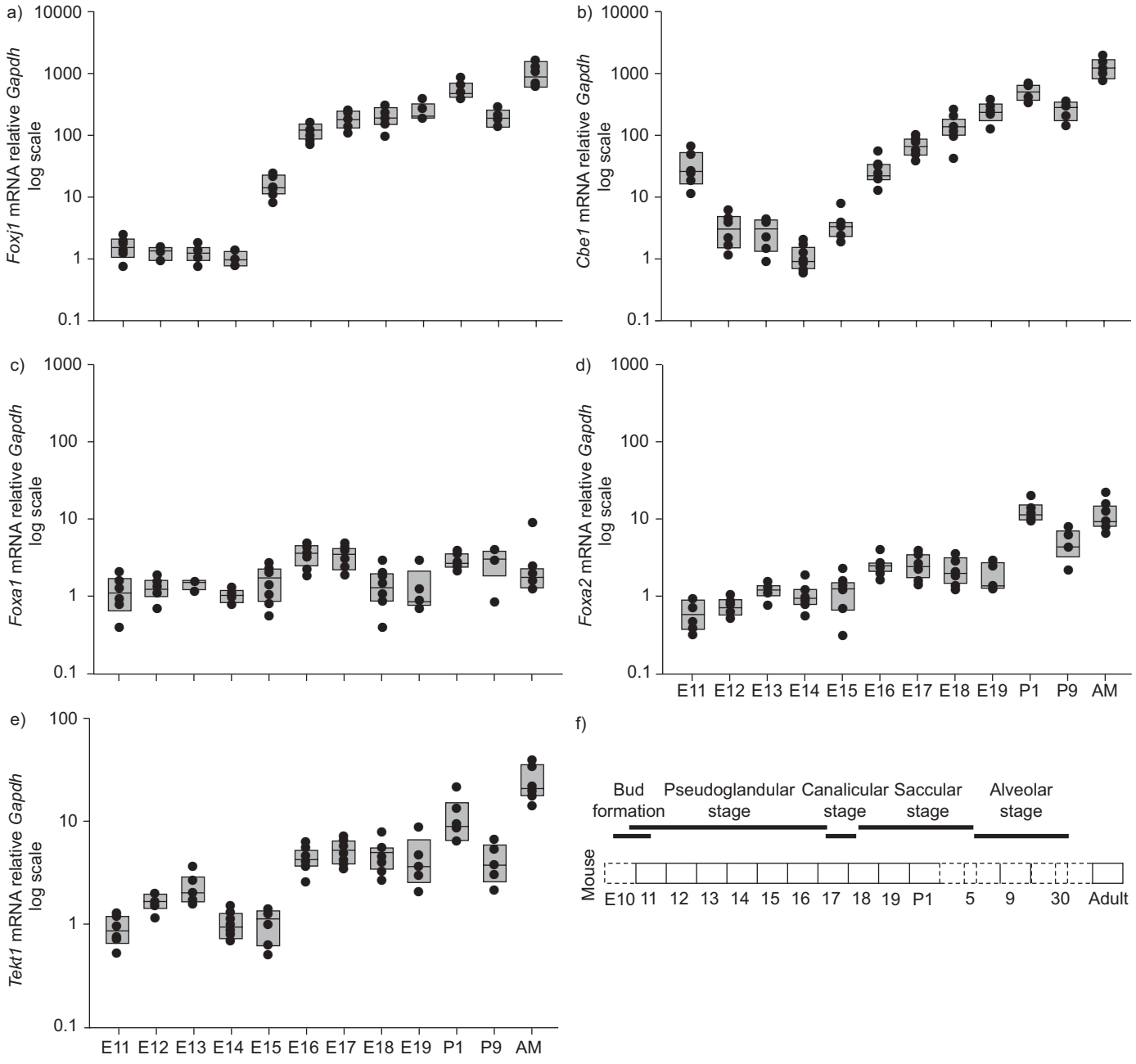

FIGURE 3. Chronological expression of a) *Foxj1*, b) *Cbe1*, c) *Foxa1*, d) *Foxa2* and e) *Tekt1* mRNAs during all stages of mouse lung development. f) Schematic representation of all stages of mouse lung development. Mouse lung tissues were dissected from embryos at the indicated days following gestation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

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, the current authors cultured embryonic/fetal lung tissues *in vitro* in order to mimic *in vivo* development and assessed the induction of *CBE1* mRNA by RT-qPCR. Prior to culture, expression of *CBE1* mRNA in embryonic lung obtained at 7–9 weeks post-conception was scarcely detectable (Ct values were ~36–40; data not shown), consistent with the semi-quantitative RT-PCR analyses (fig. 4a and 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 ~10-fold at day 12 ($p=0.01$) and day 18

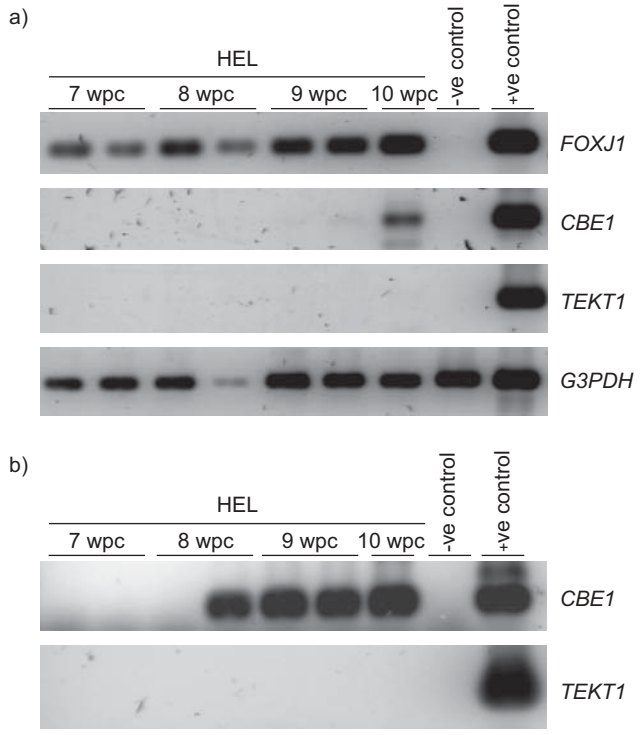

FIGURE 4. Expression of *CBE1* mRNA in human embryonic/fetal lungs (HEL). a) Detection of mRNA analysed by semi-quantitative RT-PCR. Bronchial tissues were taken from HELs at 7, 8, 9 or 10 weeks post-conception (wpc). Each lane represents a different donor. PCR cycles were 35 for analysis of *FOXJ1*, *CBE1* and *TEKT1* and 25 for *G3PDH* cDNAs. cDNA from air–liquid interface-differentiated bronchial epithelial cells (at day 14) or cDNA from cultured bronchial fibroblasts was used as positive (+ve) or negative (-ve) 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 in (a) and the respective nested primers.

($p=0.01$), compared to day 0 (fig. 6b). The increase of expression levels was greater for *CBE1* than *FOXJ1* mRNA, probably because *FOXJ1* was already significantly expressed before the start of culture at 9 weeks post-conception (fig. 4a). However, the present authors 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 and 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 (fig. 6c and d), 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. 6e). Figure 7 shows a schematic representation summarising the temporal pattern of *Cbe1/CBE1* expression in developing mouse and human lungs. Although the present authors 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 >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, the present authors 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. During the period of the experiments (up to 18 days), the present authors observed maintenance of branching morphogenesis. In terms of CBE1 protein expression, induction of expression after 18 days *in vitro* (9 weeks post-conception plus 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.

A previous study observed that transcription of *CBE1*, *FOXJ1* and TEKT1 mRNAs was synchronous during *in vitro* differentiation using an 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*, a distinct order of expression was observed during differentiation of ciliated cells in the airway epithelium where *Foxj1/FOXJ1* was earlier than *Cbe1/CBE1* mRNAs, while expression of *TEKT1* was undetectable ⩽11–12 weeks post-conception. A further difference between the human adult and embryonic tissue was the protein distribution of CBE1. In adult

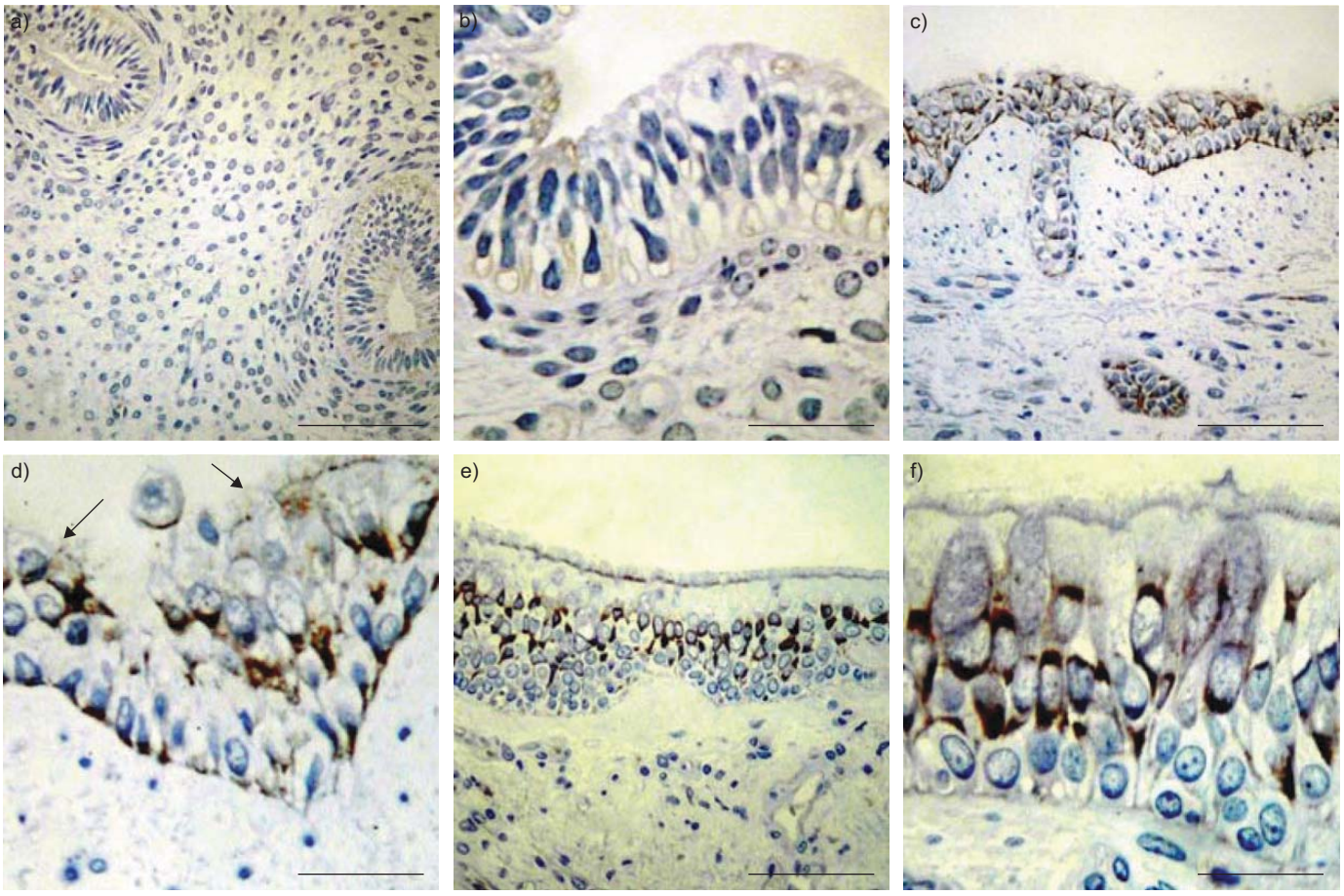

FIGURE 5. Expression of the CBE1 protein analysed by immunohistochemistry. Brown immunostaining using diaminobenzidine as chromagen shows the presence of CBE1 which is not detectable at 10 weeks post-conception (a and b), but is strong in epithelium at 12.3 weeks post-conception (c and d) where ciliary structures are visible (arrows). 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 and f). a, c and e) Scale bars=60 μm; b, d and f) scale bars=20 μm.

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 mucus-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 (figs 3 and 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* hybridisation analyses [27]. However, it should be noted that the present authors 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, the present authors were unable to confirm *CBE1* mRNA expression in human lungs as the appropriate embryonic tissues could not be obtained 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 no *TEKT1* mRNA was detected 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 E12 onwards [29]. These data suggest that the regulatory mechanisms controlling transcription of TEKT1/*Tekt1* mRNAs during embryogenesis

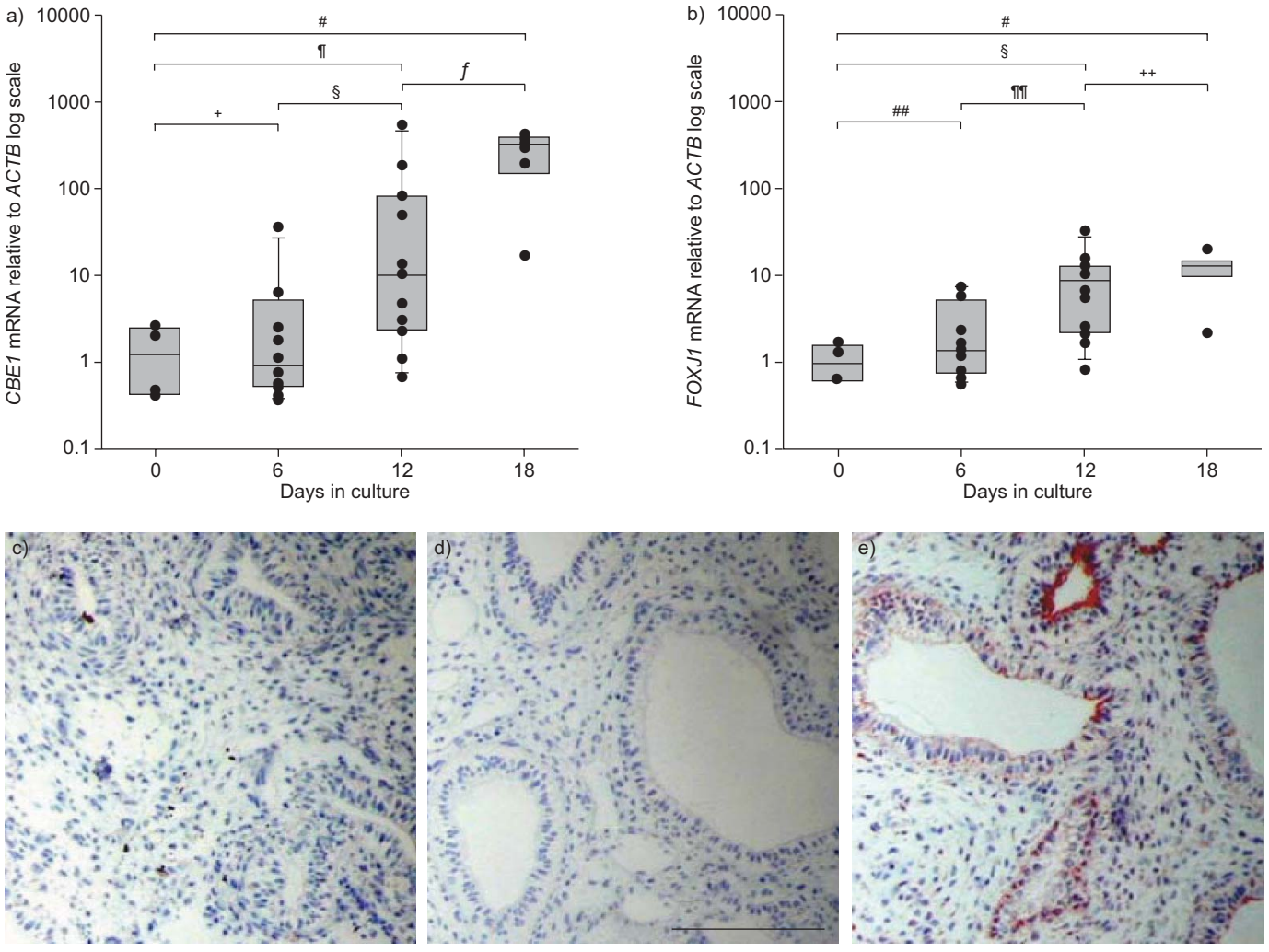

FIGURE 6. Chronological expression of a) *CBE1* and b) *FOXJ1* mRNAs during *ex vivo* differentiation of human embryonic/fetal lungs. mRNA expression was analysed by RT-quantitative PCR. Isolated human fetal lungs (7–9 weeks post-conception) were cultured and total RNA was isolated after the start of culture at the days indicated. Each data point represents the result obtained from an independent sample. Data are normalised relative to the housekeeping gene, *β-actin* (*ACTB*) mRNA levels. c–e) Protein expression was analysed by immunohistochemistry. Cultured (day 6 or 18; d and e, respectively) or noncultured (day 0; c) embryonic lung tissues were fixed and stained as in figure 5; 3-amino-9-ethylcarbazole was used as chromagen to give positive red immunostaining. Scale bar=100 μm.

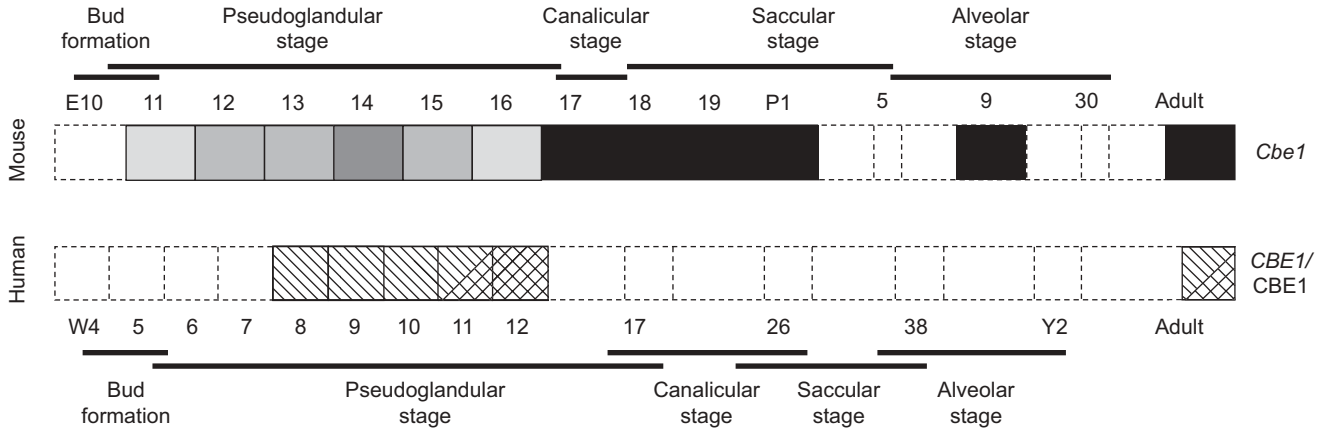

FIGURE 7. Schematic representation of stages of lung development and the temporal pattern of *Cbe1* (▫, ▪, ■ and ■) and *CBE1* (▨) mRNA expression in mouse and human lungs, and CBE1 protein (■) expression in human lungs. E: embryonic day; P: post-partum day; W: weeks post-conception; Y: year.

TABLE 1 Stages of mouse and human lung development post conception according to histological criteria

Stage	Mouse	Human
Bud formation start	~9.5 days	4 weeks (~26 days)
Pseudoglandular	9.5–16.6 days	5–17 weeks
Canalicular	16.6–17.4 days	16–26 weeks
Saccular	17.4–5 days after birth	24–38 weeks
Alveolar	Day 5–30 after birth	36 weeks to 1–2 yrs after birth

Data taken from [23–26].

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 the present study which shows that *Foxa1* and *Foxa2* mRNAs were constantly detectable in the mouse embryonic lungs at E11 and thereafter (fig. 3c and d). It has previously been 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. Recently, MATSUOKA *et al.* [31] described a spermatid specific gene, *Smrp1*, which is homologous to murine *Cbe1*. They reported three mRNA variants in the manchette, but only one of these transcripts was translated into protein. MATSUOKA *et al.* [31] also proposed that Smrp1 may be implicated in the formation of flagella or cilia constructed by tubulin.

Further functional studies are now required to define the role of Cbe1/CBE1 in lung epithelial differentiation and ciliogenesis.

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