Structural Development, Cellular Differentiation and Proliferation of the Respiratory Epithelium in the Bovine Fetal Lung

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Abstract

Foetal bovine lung samples of 11 different gestational ages were assigned to a classical developmental stage based on histological morphology. Immunohistochemistry (IHC) was used to characterise the morphology of forming airways, proliferation rate of airway epithelium and the presence of epithelial cell types (ciliated cells, club cells, neuroepithelial cells and type II pneumocytes). Typical structural organisation of pseudoglandular (84-98 days of gestational age [DGA]), canalicular (154-168 DGA) and alveolar (224-266 DGA) stages was recognised. In addition, transitional pseudoglandular-canalicular (112-126 DGA) and canalicular-saccular (182 DGA) morphologies were present. The embryonic stage was not observed. A significantly ($p<0.05$) higher proliferation rate of pulmonary epithelium, on average 5.5% and 4.4% in bronchi and bronchioles, respectively, was present in the transitional pseudoglandular-canalicular phase (112-126 DGA) compared to all other phases, whereas from 8 weeks before term (224-266 DGA) proliferation had almost ceased. The first epithelial cells identified by specific marker proteins in the earliest samples available for study (84 DGA) were ciliated cells and neuroepithelial cells. Club cells were present initially at 112 DGA and type II pneumocytes at 224 DGA. At the latest time points (224-226 DGA) these latter cell types were still present at a much lower percentage compared to adult cattle. This study characterised bovine foetal lung development by histological morphology and cellular composition of the respiratory epithelium and suggests that the apparent structural anatomical maturity of the bovine lung at term is not matched by functional maturity of the respiratory epithelium.

Introduction

The structure of mammalian lungs is highly species dependant (Plopper *et al.*, 1983; Warburton *et al.*, 2000) as are the timings and patterns of epithelial cell differentiation during gestation and postnatal development (Hyde *et al.*, 1983; Plopper *et al.*, 1992a; Plopper and Fanucchi, 2004; Plopper *et al.*, 1980a; Plopper *et al.*, 1980b). Mammalian lungs undergo five recognised morphological developmental phases from conception to parturition; embryonic, pseudoglandular, canalicular, saccular and alveolar, respectively (Burri, 1997; Corrin, 2000). In the earliest, embryonic, phase the rudimentary trachea and lungs differentiate from the endoderm of the foregut and the main conducting airways develop from the first clusters of epithelial cells. The branching of these conducting airways corresponds to the branching of the initial pulmonary blood vessels and the main lobulation of the pulmonary mesenchyme also develops at this phase. The pseudoglandular phase, named because of the morphological resemblance of airway ducts in cross-section to glandular tissue, is characterised by intensive formation of additional conducting airways which become bronchi, with associated cartilage, and bronchioles. By the end of this phase terminal bronchioles are present. The canalicular phase is characterised by narrowing of the distal ends of the terminal bronchioles and the appearance of the first airspaces which will, in the subsequent saccular phase, develop by the process of septation into sacculi (pocket-like structural units). During the saccular phase blood vessels develop a close proximity to tissue destined to undertake gaseous exchange. In the final, alveolar, phase the sacculi transform by secondary septation into millions of alveoli which are the primary units of gaseous exchange (Burri, 2006; Morrisey and Hogan, 2010). Generally, rodents are born with their lungs at the saccular phase whereas humans are born with their lungs at the alveolar phase (Pinkerton and Joad, 2000). In humans the alveolar phase first appears approximately four weeks before birth (36 of 40 weeks gestational age [WGA]); alveolarization continues postnatally for up to two years (Schittny and Burri, 2008).

In cattle a single report based on the examination of 60 bovine foetuses described the relationship between age of gestation, derived from crown-rump length and other morphological features, and lung developmental phases by histological morphology (de Zabala and Weinman, 1984). The study estimated that the alveolar phase began by the 34th WGA, which is at least six weeks prior to parturition (based on a gestation period of 280-290 days for cattle). In sheep gestation is shorter (148 days) but the alveolar phase of lung development is present four weeks before birth (Alcorn *et al.*, 1981). Therefore, for both these ruminant species the alveolar phase appears at approximately 80% of the gestation period while in humans the alveolar phase does not appear until 90% of the gestation period. Concurrent with the development of conducting airways and alveoli during gestation the respiratory epithelium differentiates into a number of highly specialised cells, with a variety of functions, that enable gaseous exchange and also protect the lung from invading pathogens and noxious agents (Knight and Holgate, 2003). The main epithelial cells present within conducting airways are ciliated epithelial cells, goblet cells, club cells (formally known as Clara cells [Winkelmann and Noack, 2010]) and basal cells (Corrin, 2000). The majority of epithelial cells found within the gaseous exchange region are type I and type II pneumocytes (Corrin, 2000). In addition single neuroepithelial cells (NEC) and clusters of several NEC, named neuroepithelial bodies (NEB), are present in both the conductive and respiratory areas of lung but in low numbers (Van Lommel, 2001).

As the rate and timing of appearance of the various respiratory epithelial cells during gestation has not been reported for cattle, the aims of this study were to relate, in bovine foetuses of varying gestational age, histological lung morphology and detailed composition of respiratory epithelium in terms of the number and distribution of the principal cell types and their proliferative activity.

Materials and Methods

Tissue Samples and Processing

A total of 15 foetuses from clinically healthy cows, used as negative control animals from experiments conducted previously (Benavides *et al.*, 2012; Macaldowie *et al.*, 2004; Maley *et al.*, 2003) were used. As determined by insemination dates, a sample from the left caudal lung lobe was collected from two foetuses each of 84, 98, 112 and 126 days of gestational age (DGA) and from one foetus of 154, 168, 182, 224, 238, 252 and 266 DGA. The samples were fixed in 10% neutral buffered formalin and processed routinely prior to embedding in paraffin wax. Sections were cut at 4µm, mounted on glass microscope slides and stained with haematoxylin and eosin prior to light microscopy.

Determination of Phase of Lung Development

Under blinded conditions each lung sample was assessed by histological morphology and assigned, where possible, to one of the five phases of development recognised in human lungs (embryonic, pseudoglandular, canalicular, saccular and alveolar) (Corrin, 2000) or to a transitional phase between two adjacent developmental phases when the morphology present was not typical of any single phase. The lung phase was then aligned, retrospectively, with the day of gestational age.

Immunohistochemistry

An anti-pan-cytokeratin antibody was used to label all the respiratory epithelial cells to visualise developing airways. Ciliated respiratory epithelial cells were labelled by targeting the beta-tubulin protein, club cells by club cell secretory protein (CCSP), type II pneumocytes by surfactant protein-C (SPC) and dendritic cell-lysosomal associated membrane protein (DC-LAMP), neuroepithelial cells by the neurotransmitter synaptophysin

and cellular proliferation by the nuclear marker of cell division Ki67 (Table 1). Lung samples from three normal calves (2-4 months old) were used as positive control sections for cell type markers. For the anti-Ki67 antibody positive control material was a bovine lymph node from a clinically healthy 2 month old calf. The negative control samples comprised semi-serial sections with the exception of having the primary antibody substituted with a species and isotype matched antibody, derived from normal sera for polyclonal antibodies, or purified immunoglobulins for monoclonal antibodies.

Lung sections (4µm thick) were mounted on charged glass slides (Superfrost Plus™ slides, Menzel-Gläser, Braunschweig, Germany), dewaxed in xylene, rehydrated through graded alcohols and washed in tap water prior to immersion in 3% H_2O_2 in methanol (v/v) for 20 min to block endogenous peroxidise activity. For antigen retrieval, sections were autoclaved in 0.01 M citrate buffer (pH=6.0) for 10 min at 121°C. Sections were subsequently washed in phosphate buffered saline (PBS) containing 0.05% $Tween_{20}$ (PBST). Non-specific antibody binding was blocked by incubation with 25% normal goat serum, diluted in PBST, for 30 min. Samples and positive and negative control sections were then incubated overnight at 4°C with the respective primary antibody (Table 1) diluted in PBST. The following day, sections were washed in PBST and the primary antibodies visualised by a commercial system (EnVision™ System-HRP, Dako, Ely, UK) using the chromagen 3,3'-diaminobenzidine tetrachloride (DAB) (EnVision™ System-HRP) according to the manufacturer's instructions. Sections were counterstained in hematoxylin "Z" (CellPath, Newtown, UK), blued in Scott's tap water substitute, dehydrated through graded alcohols, cleared in xylene and mounted using Consul-mount (Thermo Scientific Shandon, Reading, UK) prior to examination by light microscopy. Primary antibodies were validated and optimised previously by serial dilution using bovine foetal lung sections.

Quantitation and Statistical Analyses

The anatomical regions distinguished in the quantitative analysis of lung epithelial cells depended on the phase of lung development. In samples of the pseudoglandular and pseudoglandular/canalicular phases, regions of bronchi, bronchioles and immature developing ducts were described separately. For the canalicular phase regions of bronchi, bronchioles and canaliculi and for the alveolar phase regions of bronchi, all bronchioles and alveoli were distinguished. Photomicrograph images of five or 10 (for counting club cells within bronchi, small sample sizes or assessing labelling by anti-pan-cytokeratin antibody) randomly selected, non-overlapping fields from each tissue section were collected for quantification of respiratory epithelial cells and, separately, ciliated epithelial cells, club cells and type II pneumocytes. Neuroepithelial cells and NEB were counted in the whole area of the lung tissue section.

The area within each field labelled by anti-pan-cytokeratin antibody was defined as the respiratory epithelial surface area (A_{epith}, mm^2) and was expressed as a percentage of the area occupied by tissue (A_{tiss} [total area of tissues section – area of airspaces and area of blood vessel lumina]) in the field ($Epith_{\%} = (A_{epith} / A_{tiss}) \times 100$) (%). Additionally, in each photomicrograph, the surface area of the airspaces (ASA) was calculated and expressed as a percentage of A_{tiss} and the number of airways was calculated per mm^2 of lung section. Ciliated cells, as denoted by labelling of beta-tubulin, could not be distinguished as single labelled cells in most cases so the area labelled by anti-beta-tubulin antibody was expressed as a percentage of the respiratory epithelial surface area ($\beta\text{-tubulin}_{\%,epith}$)(%). Club cell numbers were counted directly and the final values were expressed as a percentage of all bronchial and bronchiolar respiratory epithelial cells examined in the photomicrograph. Type II pneumocytes were counted within alveolar regions and, similarly, the percentage of positive cells relative to all respiratory epithelial cells was calculated. The paucity of NECs

and NEBs dictated that they be counted in the whole tissue sections and their quantities expressed as total/mm^2 of lung tissue. The proliferation rate of respiratory epithelium was described by the cell proliferation index (CPI), namely the number of proliferating cells, as denoted by Ki67 positive labelling, expressed as a percentage of the respiratory epithelial cells examined. The mean values of CPI were calculated for each of the developmental phases analysed. All measurements and calculations in photomicrographs were performed using Image J software (Rasband, 1997-2011).

Changes in numbers of each epithelial cell type studied were analysed by descriptive statistics only due to the restricted numbers of samples. In all analyses, the mean and minimum-maximum values were used to summarise the location and variability in the data. A REML analysis (GenStat 15th edition) was used to analyse differences between the CPIs at different developmental phases, for bronchi and bronchiolar regions separately. Data were analysed by fitting mixed models which incorporated animal as a random effect and developmental phase as a fixed effect to arc-sine transformed data. Statistical significance for the fixed effect was estimated using the Wald statistic. Comparisons between developmental phases were adjusted for multiple testing using the False Discovery Rate method (Benjamini and Hochberg, 1990). To estimate trend lines for the increase in beta-tubulin amount in bronchial/bronchiolar epithelium with gestational time, additive models were fitted to the data using cubic splines, with the level of smoothing specified using generalized cross validation. This was implemented using the smooth.spline function from the stats package in R version 3.0.2.

Results

Determination of Phase of Lung Development

Haematoxylin and eosin stained sections, along with pan-cytokeratin labelled semi-serial sections, enabled the morphological classification of each bovine foetal sample into appropriate developmental phases and showed the extent of the respiratory epithelium development with gestational age (Fig.1, Fig.2). None of the foetal bovine lung samples examined exhibited the morphology of the embryonic phase. The earliest gestational age available to be examined, 84 DGA (n=2), showed typical pseudoglandular phase morphology with conducting airway ducts at different developmental stages surrounded by large amounts of mesenchyme containing developing vasculature, including lymphatics (Fig.1a). Transverse-sections of conducting airways showed different structural organisations. Airways associated with cartilage were defined as bronchi, those surrounded by smooth muscle layers only and no cartilage were classified as bronchioles and the rest were comprised of round ducts without smooth muscle tissue. These less developed airways varied in size but were all less than 100 μm in diameter. The least well developed were devoid of a lumen and filled completely by epithelial cells representing the most distal parts of the developing respiratory tract. Similar tissue structure was observed in lungs from 98 DGA but the number of airways was greater and the mesenchymal tissue less abundant (Fig.1b).

Samples at 112 and 126 DGA were classified as at a transient phase between pseudoglandular and canalicular because the first air spaces, lined by squamous epithelium, were present at the distal end of conductive airways. The mesenchymal tissue was more cellular (Fig.1c) and the vascular system was more extensively developed. In samples of 154 and 168 DGA, typical canalicular phase morphology was present since no new developing conductive airways were observed and cell density within the mesenchyme had increased further (Fig.1d). At 182 DGA the beginning of septation within air spaces was present along with the simultaneous presence of canaliculi at the end of some terminal bronchioles, therefore, this morphology was judged to represent the transition between canalicular and saccular phases of foetal lung

development (Fig.1e). The classical morphology of the saccular phase was not present in any of the samples available for examination. The remaining samples examined (224, 238, 252 and 266 DGA) were all at the alveolar phase of lung development. These lung samples consisted of well-developed respiratory bronchioles, alveolar ducts and alveoli. The respiratory epithelium of the alveoli consisted of a dense concentration of cells organised primarily in single layers that remained in direct contact with blood and lymphatic vessels (Fig.1f, 1g). In summary, by morphology, the embryonic phase of lung development in foetal cattle was completed at some point prior to 84 DGA as by this time the pseudoglandular phase was well established. The pseudoglandular phase was completed by 112 DGA and a transitional phase existed until at least 126 DGA. The canalicular phase of lung development was well established by 154 DGA and continued to at least 168 DGA. By 182 DGA the saccular phase was commencing. We were unable to determine the length of duration of the saccular phase as by day 224 DGA the alveolar phase was well established.

Development of Respiratory Epithelium

The pan-cytokeratin labelled sections showed clearly the extent of development of the respiratory epithelium at each available time-point (Fig.2). The epithelium of the bronchi was pseudostratified in the pseudoglandular phase (Fig.2a, 2b). However, the bronchiolar epithelium was comprised of simple columnar cells with apically located nuclei and contained a single large, empty vacuole within the cytoplasm (Fig.2c). Developing airways were lined mostly by columnar epithelium but stratified, cuboidal cells tended to characterise the most distal regions. In some places, groups of pan-cytokeratin labelled cells, without any airway organization, were present (Fig.2d). In lung samples of 154-266 DGA anti-pan-cytokeratin antibody labelled primarily regions of respiratory epithelium with the closest proximity to airspaces in the canaliculi, sacculi and alveoli (Fig.2e, 2f).

237 Quantitative analysis of respiratory epithelial surface area (A_{epith}) in lung samples of different
238 gestational age was performed only for the earliest phases (84-126 DGA) as inconsistent pan-
239 cytokeratin labelling in the later canalicular and alveolar phases precluded analysis of later
240 phases. Each time point analysed was represented by 2 animals. Over the first three time
241 points (84-112 DGA) $Epith_{\%}$ increased from 4.5% to 9.4%. At 126 DGA $Epith_{\%}$ was 6.1%
242 (Fig.3). The number of developing airways per mm^2 increased from 21.2 at 84 DGA, to 81.5
243 at 126 DGA (Fig.3). Despite an increasing number of airways with gestational age ASA
244 remained relatively constant for all animals examined and ranged between 0.8-2.5% (Fig.3).
245
246 *Differentiation of Ciliated Epithelial Cells*
247 The earliest age at which cilia were identified on respiratory epithelial cells, as indicated by
248 labelling of beta-tubulin, was 84 DGA and although present in both bronchi and bronchioles
249 they were relatively rare (Fig.4a). The number of ciliated cells increased with gestational age
250 (Fig.4a, 4b). In bronchioles the number of ciliated respiratory epithelial cells decreased with
251 proximity to the alveolar region, but were present up to respiratory bronchioles (Fig.4c). In
252 early gestation (84-112 DGA) most of the beta-tubulin was present throughout the cytoplasm
253 of the cells whereas in later phases it was present predominantly in the apical part of the cell
254 cytoplasm and the cilia. Some labelling was also present within surrounding sub-epithelial
255 tissue but the strongest labelling clearly occurred in epithelial cells and their cilia.
256 Quantitative analysis demonstrated that beta-tubulin ($\beta\text{-tubulin}_{\%,epith}$) was present in 12.8% to
257 27.6 % of the epithelial surface of bronchi and 9.1 % to 26.8 % of bronchioles (Fig.4d). A
258 trend for increasing amounts of beta-tubulin ($\beta\text{-tubulin}_{\%,epith}$) with gestational age was
259 observed throughout gestation in bronchiolar epithelium. However, in bronchial epithelium it
260 was present only in animals between 84 DGA to 168 DGA (Fig. 4d). Later in gestation the
261 quantity of this protein was variable (Fig. 4d).

Club Cell Differentiation

Within the bronchial epithelium, the first CCSP-positive cells were present in only one of the two samples from 112 DGA (Fig.5a, 5b). In bronchioles, club cells were first recognised at 154 DGA. Initially, labelled club cells were present within the airway epithelium of bronchi and bronchioles as a small proportion of the epithelial cells (<5% at 112-182 DGA) but this increased gradually with gestational age in both bronchi and bronchioles (Fig.5b). In the alveolar phase (224-266 DGA) club cells comprised 27.5 % and 11.6 % (mean values) of bronchial and bronchiolar epithelial cells respectively (Fig.5b). However, in the terminal bronchioles the number of club cells remained low (<1% of epithelial cells) at all gestational time points examined. No CCSP-positive cells were present in any of the alveolar regions. The sample of 168 DGA was excluded from analysis due to inconsistent labelling of club cells on repeated immunohistochemistry attempts.

Type II Pneumocyte Differentiation

Both type II pneumocyte cell marker proteins, SP-C and DC-LAMP, were detected initially at 112 DGA as abundant, punctate, cytoplasmic labelling within cells filling the lumina of immature, developing airway ducts (Fig.6a). Subjectively, a decrease in the number of cells with this labelling pattern appeared to occur with increasing gestational age, such that they were present only occasionally in the canalicular phase, usually in areas adjacent to developing air spaces. From the earliest sample available in the alveolar phase (224 DGA) both SP-C and DC-LAMP positive cells were present as single, isolated cells, all of which were within the alveolar region and typically in the corners of alveoli (Fig.6b, 6c). On average, 10.5% of cells within the alveolar region were labelled for DC-LAMP and 5.4% for

SP-C from samples between 224-266 DGA (Fig.6d). Bronchi and bronchioles were devoid of any SP-C and DC-LAMP labelling in all samples examined.

Distribution of Neuroepithelial Cells and Bodies

Neuroepithelial cells (NEC) and NEB, as represented by labelling of synaptophysin (Gould *et al.*, 1986) (Fig.7a), were present in all samples but only in small numbers. The number of NEC per mm^2 of lung section was higher than for NEB in each sample examined. At 84 DGA the majority of NEC and NEB were within bronchi and bronchioles. At 98-126 DGA NEB were present equally in developing airways at different developmental stages, but NEC were mostly associated with bronchi. In the later phases (alveolar) of bovine foetal lung development NEC/NEB were found in conductive airways and alveolar regions of the lung but at low density. The greatest density of NEC was found in one animal at 84 DGA and of NEB in one animal at 98 DGA, at 0.68 and 0.23/mm^2 of lung section, respectively (Fig.7b). The density tended to decrease with gestational age for both NEC and NEB to 0.01/mm^2 of lung section at the later gestational time points (252-266 DGA). In samples from 154, 168, 238 and 252 DGA NEB were not found.

Proliferation Rate of Respiratory Epithelium

For bronchial epithelium the mean CPI, as denoted by positive labelling of cells for Ki67, ranged from 6.7 % (126 DGA) to <0.1% (238 and 266 DGA) of cells within all sections examined. For one animal (168 DGA) no bronchi were present in the semi-serial sections used due to progression through the tissue block and for two samples (98 DGA, and 252 DGA) no bronchi were present in the sample. The highest mean CPI within the bronchial respiratory epithelium occurred in the transient, pseudoglandular-canalicular phase (112 and 126 DGA) and was 5.5 % (mean value for all samples). In the pseudoglandular and alveolar

311 phases (84-98 and 224-266 DGA, respectively) the mean CPI of the bronchial respiratory
312 epithelium was the lowest, 0.4% and 0.6%, respectively. However, samples within the
313 alveolar phase of development had a relatively wide range of mean CPI; 2.6% (224 DGA)
314 versus <0.1% for the rest of samples (238, 252, 266 DGA) (Fig.8).
315 In the respiratory epithelium of the bronchioles, the mean CPI ranged from 5.7% (112 DGA)
316 to <0.1% (154, 182, 238, 252, 266 DGA). However the number of time points with a very
317 low CPI was greater than for the bronchi. The developmental phase with the highest mean
318 CPI in the bronchiolar regions was the transient pseudoglandular-canalicular phase with a
319 CPI of 4.4%. In the alveolar phase the samples from the last three time points had CPIs of
320 <0.1% and the mean CPI for this phase was the lowest of all developmental phases examined
321 (0.3%) (Fig.8).
322 The epithelium of distal developing airways could be recognised in bovine foetal lungs
323 between 84-126 DGA. The mean CPI of epithelial cells in this anatomical area ranged from
324 0.5 - 10.1%. This latter value was the highest mean CPI observed in all anatomical regions
325 and samples analysed and was found at 112 DGA which is during the transition from the
326 pseudoglandular to the canalicular phase. In samples from the canalicular phase, canaliculi
327 could be differentiated from bronchi and bronchioles but the CPI in their epithelium could not
328 be calculated because it was not possible to accurately differentiate the epithelial cells from
329 adjacent mesenchymal cells. In the alveolar phase differentiation of epithelial cells from
330 mesenchymal cells was possible and the mean CPI in the earliest stage of the alveolar phase
331 available for examination (224 DGA) was 1.3% but in the subsequent three time points (238,
332 252, 266 DGA) the mean CPIs were consistently <0.1 % (Fig.8).
333 Statistical analysis of the respiratory epithelial proliferation rate in samples grouped
334 according to their developmental phase (pseudoglandular, transient pseudoglandular-
335 canalicular, canalicular and alveolar) confirmed that the proliferation rate varied significantly

between groups in both the bronchial epithelium (Wald statistic; W=66.21, p=0.002) and the bronchiolar epithelium (Wald statistic; W=27.17, p=0.005). For both anatomical regions, the transient pseudoglandular-canalicular phase had the highest mean CPI values (bronchi: CPI =5.5%, bronchioles: CPI=4.4%); the mean value for bronchial epithelium in this phase was statistically significantly greater than those at the other three phases, in each case at a false discovery rate of less than 0.1%. The mean value for bronchiolar epithelium in this phase was statistically significantly greater than those at the pseudoglandular and alveolar phases, in each case at a false discovery rate of less than 0.1%. There was, however, no statistically significant difference between the mean proliferation rates at the transient pseudoglandular-canalicular and canalicular phases. In summary, the CPI of respiratory epithelial cells in the bronchi and bronchioles was maximal during the transition between the pseudoglandular and canalicular phases of bovine foetal lung development but proliferation had almost stopped during the alveolar phase in all the anatomical regions examined (bronchi, bronchioles and alveoli).

Discussion

This is the first study to determine the morphological phase of bovine foetal lung development at precisely known gestational ages and also the first to investigate the times of appearance, location and changes in relative numbers of pulmonary epithelial cells during different periods of gestation. The results are summarised schematically in Fig.9.

Our findings show that in cattle the final, alveolar, phase of lung development begins approximately 8 weeks prior to birth, which is relatively early compared to other mammalian species (Pinkerton and Joad, 2000) but similar to other ruminants (Alcorn *et al.*, 1981; de Zabala and Weinman, 1984). This relative lung maturity is presumed beneficial in helping new-born wild bovids to rapidly achieve proficient locomotion in order to remain with the

361 herd and avoid predators. The alveolar phase is also associated with a dramatic reduction in
362 cell proliferation suggesting the lungs are probably as developed as they can be *in utero* some
363 time prior to parturition. With respect to gestational age and morphological development our
364 findings are broadly in agreement with a previous study (de Zabala and Weinman, 1984).
365 However, development of the alveolar phase was found earlier in the present study; 224 DGA
366 compared to 240 DGA. This difference may be due to the earlier study using crown-rump
367 length and other gross morphological features to determine DGA which is less accurate than
368 date of artificial insemination, especially in the last trimester of pregnancy (Anderson, 2012).
369 Notably, gestation length is influenced by breed in cattle (Noakes, 1986) and also weather
370 conditions (Troxel and Gadberry, 2012).
371 A functional respiratory system requires not only the mature physical morphology of the
372 lungs but also the appropriate cell phenotypes to be present in the correct numbers and
373 locations. Ciliated epithelial cells are the most numerous epithelial cell in the bronchi and
374 large bronchioles of postnatal mammals (Harkema *et al.*, 1991) and are one of the first cells
375 to morphologically mature in the respiratory epithelium (Plopper *et al.*, 1992a). In our study
376 they were present in small numbers in both bronchi and bronchioles by 84 DGA, which is
377 approximately 30% of gestation in cattle. Although this was the earliest time point available
378 for examination the low numbers suggest this may correspond to the first appearance of these
379 cells. This time of appearance is comparable with humans (Jeffery *et al.*, 1992) but much
380 earlier compared to small rodents where ciliated epithelial cells do not appear until 70-80% of
381 gestation (Plopper *et al.*, 1992a). Notably, neonatal rodents are born hairless and blind and do
382 not leave the nest for some time. Martineau et al. (2013) did not find ciliated cells until 140
383 DGA in sheep (95% of gestation) but beta-tubulin positive cells were present from 67 DGA,
384 (46% of gestation). Why cilia were so late to develop in sheep despite the presence of beta-
385 tubulin positive cells is unknown.

386 The number of ciliated epithelial cells increased rapidly with gestational age until 224 DGA
387 when they were the most prominent cell in bronchial and bronchiolar epithelium. However,
388 the subjective observation of a gradual increase in the number of ciliated cells with
389 gestational age in both bronchi and bronchioles did not correspond well with the beta-tubulin
390 content in the respiratory epithelium ($\beta\text{-tubulin}_{\%,epith}$) which showed high variability in the
391 last trimester (182-266 DGA). Studies on sheep showed that during postnatal development
392 the absolute amount of beta-tubulin in the respiratory epithelium significantly increased in the
393 bronchi and bronchioles (Martineau *et al.*, 2013) so the amount of beta-tubulin can be up-
394 regulated periodically. Therefore, caution should be exercised in using quantitative estimates
395 of beta-tubulin protein to determine the extent of ciliation present in respiratory epithelium.
396 Pan-cytokeratin, similarly to beta-tubulin, did not reflect, quantitatively, the increase in
397 airway epithelium during gestation. The pan-cytokeratin labelled epithelial surface area
398 fraction ($Epith_{\%}$) remained relatively constant in the lung sections examined, presumably due
399 to development of progressively more distal airways of smaller diameter comprised of
400 attenuated epithelial cells which less surface area. Additionally, the airspace surface area
401 remained relatively constant (0.8-2.5%), presumably reflecting that during early gestational
402 phases (84-126 DGA) many of the developing airways were not yet patent and luminal
403 airspaces could be observed only in the largest ones.
404 Club cells, as denoted by labelling with anti-CCSP antibody, first appeared in the foetal
405 bovine lung at 112 DGA in the bronchi and at 154 DGA in the bronchioles. Whilst this
406 timing is broadly comparable to humans (105 DGA) (Barth *et al.*, 1994) the spatial pattern of
407 cell differentiation appeared to differ between humans and cattle. Barth et al. (1994)
408 identified the initial appearance of club cells (by IHC for CCSP) in small clusters within
409 bronchioles whereas in the present study we identified only single club cells in the bronchi of
410 bovine foetal lungs. In human foetal lungs at 168 DGA the percentage of club cells was

411 approximately twice as many in the epithelium of bronchioles (11.2%) compared to bronchi
412 (5.4%). Whereas in bovine foetal lung samples at a comparable time point (182 DGA) club
413 cells were present in a similar but inverse ratio and relatively fewer in number (1.6% of
414 bronchiolar and 3.0% of bronchial epithelium). In the alveolar phase (224-226 DGA) in cattle
415 club cells comprised, on average, 27% and 12% of bronchial and bronchiolar epithelial cells
416 respectively, and were more abundant in bronchi than bronchioles at all time points
417 examined. Conversely, the epithelium of the terminal bronchioles of bovine foetuses was
418 comprised of relatively few (<0.1%) club cells. These findings in bovine foetal lungs were
419 unexpected as in most adult mammals the highest proportion of club cells are found in the
420 bronchioles and terminal bronchioles and in adult cattle is estimated to be 50% (Plopper *et*
421 *al.*, 1980b). This suggests differentiation of club cells is not complete by parturition and
422 substantial differentiation occurs postnatally. No extensive reports on club cell differentiation
423 in the foetal lung of ruminants exists and one study in sheep with a severely restricted sample
424 size failed to find any CCSP-labelled cells in any prenatal samples (Martineau *et al.*, 2013).
425 Type II pneumocytes were labelled by two different marker antigens with different cellular
426 functions (SP-C and DC-LAMP). In mature type II pneumocytes DC-LAMP is a membrane
427 component of lamellar bodies and is also present in the cell membrane (Salaun *et al.*, 2004).
428 Whereas SP-C is a protein stored within lamellar bodies and released onto the epithelial
429 surface as a component of lung surfactant (Weaver, 1998). Both proteins were found initially
430 at 112 DGA, the pseudoglandular-canalicular phase. They appeared in the respiratory
431 epithelium of immature developing ducts as multiple small intracytoplasmic granules. This is
432 in agreement with studies in foetal lungs in humans (Khoor *et al.*, 1994) and rhesus monkeys
433 (Ten Have-Opbroek and Plopper, 1992) which detected SP-B and SP-C proteins in the
434 epithelium of future conducting airways in both the embryonic and pseudoglandular phases.
435 Despite the expression of typical markers of type II pneumocytes in the early phases of foetal

436 lung development, it has been suggested that these cells may then differentiate into multiple
437 epithelial cell types thus confounding attempts to map the development of type II
438 pneumocytes throughout gestation. Additional markers, specific for fully differentiated type
439 II pneumocytes, would be desirable. The requirement for components, one of which is to
440 reduce surface tension of the postnatally expanded lung, is, as yet unknown. However, cells
441 with extensive cytoplasmic labelling by anti-SPC and anti-DC-LAMP antibodies were
442 present at the beginning of alveolar phase (224 DGA) in our study. From this point forward
443 SP-C and DC-LAMP was restricted to cells in the alveolar region with morphology typical of
444 type II pneumocytes and considered specific for this cell type. In all the alveolar phase
445 samples examined (224-226 DGA) type II pneumocytes, labelled by DC-LAMP and SP-C
446 markers, comprised, on average, 10.5% and 5.4% of cells within the alveolar region,
447 respectively. For both markers, variation between animals was low (8.4-12.3% for DC-
448 LAMP and 4.0-6.7% for SP-C). The greater number of DC-LAMP-positive cells compared to
449 SP-C-positive cells suggests not all type II pneumocytes may be producing or storing SP-C in
450 the foetal bovine lung. This is supported by previous studies that estimated the number of
451 type II pneumocytes by their morphology by their ultrastructural morphology in sheep, at
452 birth, as approximately 30% of alveolar epithelial cells (Flecknoe *et al.*, 2003; Sozo *et al.*,
453 2006). The same studies showed a postnatal increase in ovine type II pneumocytes to
454 approximately 50% by 2 weeks of age, which was maintained in adults. In human adults and
455 rodents, type II pneumocytes comprise approximately 60% of alveolar epithelial cells and
456 (Crapo *et al.*, 1983). This suggests that bovine foetal lungs probably undergo a rapid increase
457 in the number and proportion of this cell type postnatally, however this requires confirmation.
458 NECs and NEBs are described as one of the first cells to differentiate within human
459 respiratory epithelium (Domnik and Cutz, 2011) and is consistent with the present findings in
460 bovine foetal lungs. This can be attributed to their production of several essential growth

461 factors which are required for lung development (Hoyt *et al.*, 1991; Van Lommel, 2001). In
462 bovine foetal lung samples the density of both NEC and NEB decreased with gestational age.
463 A similar decrease in density was found in other species (cat, rabbit, rat, and hamster) in early
464 postnatal life (Hoyt *et al.*, 1993; VanLommel and Lauweryns, 1997).
465 Cell proliferation was greatest in bovine foetal lung during the transition between the
466 pseudoglandular and the canalicular phases (112-126 DGA). This occurred in all of the
467 anatomical regions examined: bronchi, bronchioles and developing distal respiratory ducts
468 and suggests rapid development of these structures during this time. This phase coincides
469 with the intensive formation of both the conductive and gaseous exchange compartments
470 (Schittny and Burri, 2008) and also had the highest variation in CPI values of single animals
471 and lung anatomical regions. Some of the variation may be explained by the proximal-distal
472 pattern of development of conductive airways, although it has been reported that proliferation
473 of respiratory epithelial cells is also dependent on proximity to neuroepithelial bodies (NEB)
474 and their release of growth factors locally (Hoyt *et al.*, 1991). The CPI in the respiratory
475 epithelium of bovine lung samples decreased in the second half of gestation and was almost
476 zero by six weeks before term. A similar change has been observed in sheep (Martineau *et*
477 *al.*, 2013; McDougall *et al.*, 2011). These findings support the suggestion that the bovine
478 foetal lung attains anatomical maturity at approximately six weeks before birth. The extent to
479 which such maturity is matched by functional maturity is unknown, however it is recognised
480 that significant development continues postnatally (Castleman and Lay, 1990). Progressive
481 differentiation of lung epithelial cells and increased production of their functional proteins in
482 postnatal life has been documented in sheep (Flecknoe *et al.*, 2003; Martineau *et al.*, 2013)
483 and other species (Coppens *et al.*, 2009; Fanucchi *et al.*, 1997; Plopper *et al.*, 1992b; Plopper
484 *et al.*, 1993). However, no such a study on postnatal lung epithelium development has been
485 performed in cattle.

In summary, in the present study the morphological development of the respiratory epithelium was characterised in bovine foetuses in terms of the proportion and disposition of ciliated epithelial cells, club cells, type II pneumocytes and neuroepithelial cells and bodies. These data, when viewed in the context of reports relating to postnatal lung development in cattle (Mariassy *et al.*, 1975; Plopper *et al.*, 1980b), suggest that the apparent structural anatomical maturity of the bovine lung at term is not matched by functional maturity of the respiratory epithelium and significant postnatal maturation remains to be completed. Indeed, the apparent low percentage of club cells and surfactant positive cells in cattle at birth may be significant with respect to the high incidence of neonatal and calf pneumonia and further studies on this relationship are warranted.

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646 **Figure Legends**

647 **Figure 1**. Morphology of foetal bovine lung tissue in different stages of gestational
648 development - haematoxylin and eosin staining. a-pseudoglandular stage at 84 DGA, b-
649 pseudoglandular stage at 98 DGA. Note increasing airway density and different
650 developmental morphology between them. c-transition between pseudoglandular and
651 canalicular at 126 DGA. Note first airspaces at the distal ends of terminal bronchioles and the
652 presence of greater numbers of cells within mesenchymal tissue surrounding conductive
653 airways. d-canalicular stage at 168 DGA. Note very high density of cells in mesenchymal
654 tissue and widespread presence of canaliculi which will develop into alveolar ducts and
655 alveoli. e-transition between canalicular and saccular stages at 182 DGA. Note first sacculi
656 that arise after primary septation of canalicular units. f- alveolar stage at 266 DGA. g-alveolar
657 cell organization at 266 DGA. Note dense organization of cells within alveolar septa.
658

659 **Figure 2**. Morphology of foetal bovine lung tissue in different stages of gestational
660 development – immunohistochemical labelling of pan-cytokeratin in the respiratory
661 epithelium (brown pigment). a- epithelium of bronchi at 98 DGA. b- epithelium of bronchi at
662 126 DGA. Note well-developed pseudo-stratified organization of cells and frequent presence
663 of a single large cytoplasmic vacuole (presumed glycogen). c- epithelium of early conductive
664 airways at 84 DGA. Note columnar morphology of epithelial cells and extremely apical
665 position of nuclei. d- development of new airways at 112 DGA. Note first pan-cytokeratin
666 labelling of groups of cells within mesenchyme even before structural organisation of airway
667 present. e- canalicular structures at 182 DGA. f- alveolar epithelium at 224 DGA. Note pan-
668 cytokeratin is mostly present on the surface of cells.
669

Figure 3. Development of epithelial surface and airway surface area in bovine foetal lung at early stages of gestation. ASA- airspace surface area (%); $Epith_{\%}$- the area within each field labelled by anti-pan-cytokeratin antibody expressed as a percentage of the area occupied by tissue (A_{tiss}) (%). N – number of airways per mm^2 of lung section. Each data point represents the mean value obtained for one animal. DGA – days of gestation age. Trend line = number of airways in mm^2 of lung section and was calculated by the least squares method.

Figure 4. Ciliated epithelial cells as denoted by immunohistochemical labelling of beta-tubulin (brown pigment). a- ciliated epithelial cells in bronchi at 84 DGA. Note only a small number of cells within the respiratory epithelium have cilia on their surfaces at this stage. b- bronchi at 224 DGA. Most of the beta-tubulin labelling is associated with dense ciliation of the apical surfaces of epithelial cells. c- ciliated epithelial cells in terminal bronchioles at 224 DGA. Note small numbers, d- the area labelled by anti-beta-tubulin antibody expressed as a percentage of the respiratory epithelial surface area ($\beta\text{-tubulin}_{\%,epith}$)(%) in bronchi (red circles) and bronchioles (blue diamonds) from bovine foetuses of different gestation ages. 252 DGA and one animal of 98 DGA- no bronchi were present in these lung sections. Trend lines were estimated using cubic spine-based additive models with the level of smoothing specified using generalized cross validation.

Figure 5. Club cells as denoted by immunohistochemical labelling of club cell secretory protein (brown pigment). a- club cells in bronchi at 112 DGA. b- proportion of club cells within the epithelium of bronchi (orange circles) and bronchioles (green squares) in bovine foetuses of different gestational ages. The bars represent mean values and error bars depict minimum-maximum range. 252 DGA- no bronchi were present in the lung section. 126a and 126b- different animals of the same gestational age.

695

696 **Figure 6**. Type II pneumocytes as denoted by immunohistochemical labelling of surfactant
697 protein C (SP-C) or dendritic cell-lysosomal associated membrane protein (DC-LAMP)
698 (brown pigment). a- granules of SP-C protein in the lumina of developing airways at 112
699 DGA. Note this marker of type II pneumocytes was widespread in epithelial cells of early
700 developing airways. b- type II pneumocytes labelled by anti-SP-C antibody at 238 DGA, c-
701 type II pneumocytes labelled by anti-DC-LAMP antibody at 266 DGA. Note in the alveolar
702 phase that both marker proteins of type II pneumocytes are present only in isolated single
703 cells within the alveolar region. d- percentage of type II pneumocytes, as labelled by SP-C
704 and DC-LAMP markers, within the alveolar region at the alveolar stage of bovine foetal lung
705 development (DGA 224-266). The bars represent mean values and error bars depict
706 minimum-maximum range.

707

708 **Figure 7**. Neuroepithelial cells (NEC) and bodies (NEB) as denoted by
709 immunohistochemical labelling of synaptophysin (brown pigment). a- neuroepithelial body
710 (NEB) labelled within the epithelium of a developing distal airway at 126 DGA. b- mean
711 number of NEC (red squares) and NEB (green circles) per mm^2 of total lung tissue in animals
712 of different gestational ages. Trend lines were generated by joining mean values for numbers
713 of NEB/NEC in gestational time points examined.

714

715 **Figure 8**. Cell proliferation index (CPI), derived from immunohistochemical labelling of Ki-
716 67, of bovine lung epithelium at different phases of foetal lung development. Data are
717 depicted according to anatomical site, namely bronchi (●), bronchioles (■), distal developing
718 airways (▲) and alveolar regions (♦). The figures represent means and vertical segments

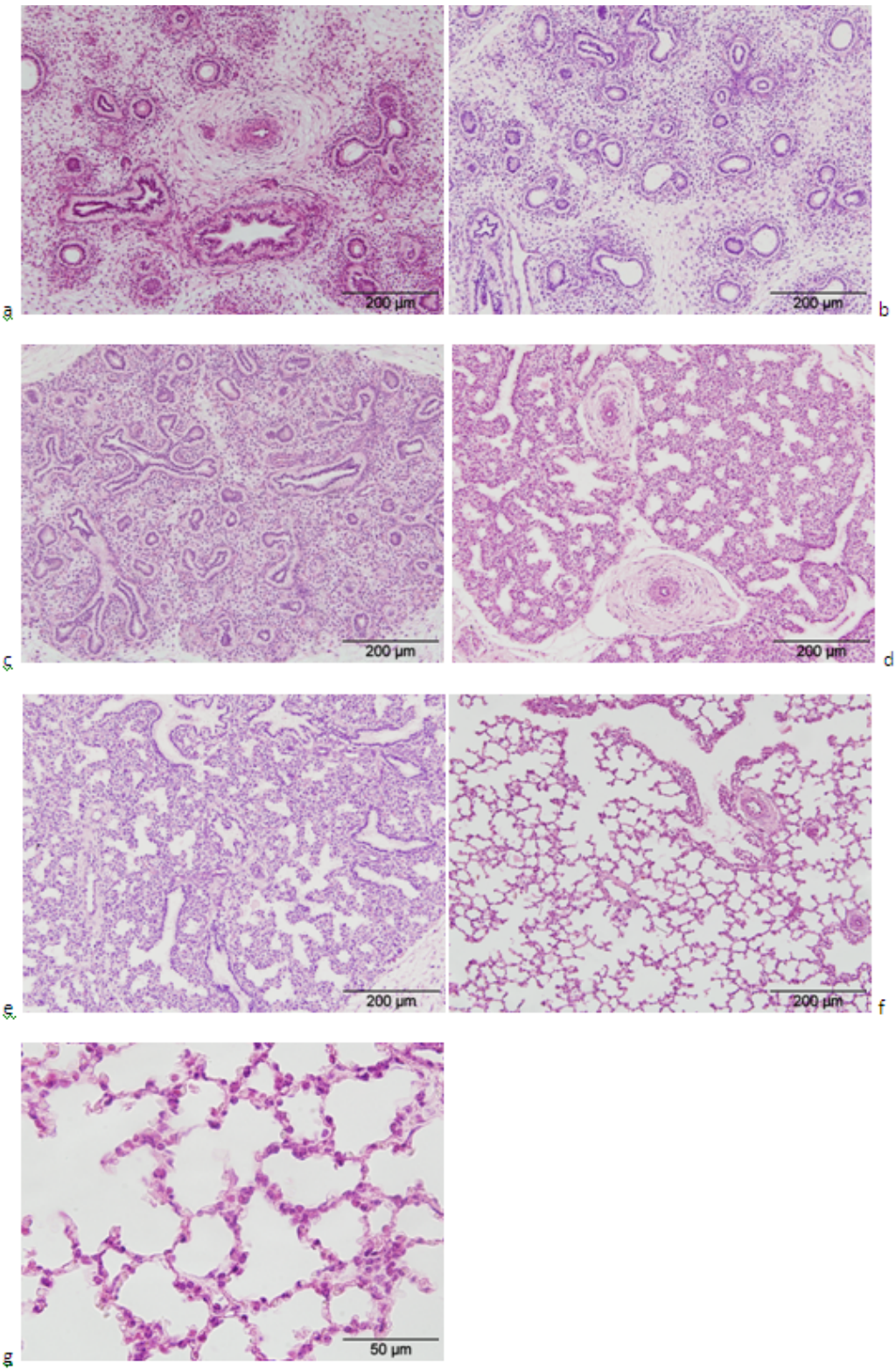
depict minimum-maximum range. a,b- different animals of the same gestational age. 98a DGA, 168 DGA and 252 DGA- no bronchi were present in the lung sections.

Figure 9. Diagrammatic representation of relative changes in different types of bovine pulmonary epithelial cells from day 84 until day 266 of gestation. Note, some changes are subjective (ciliation), some represent percentage within epithelium (type II pneumocytes, club cells) others represent density (NEB/NEC). P-pseudoglandular, C-canalicular, S-saccular and A-alveolar.

Table 1. Antibodies used for detection of different types of respiratory epithelial cells and proliferating cells.

Epithelial cell	Marker antigen	Antibody type and source	Optimal dilution	Negative control
Ciliated cells	Beta-tubulin	Anti-beta-tubulin, mouse monoclonal, clone 3F3-G2, IgM, Abcam (ab40862)	1: 60,000	Normal mouse IgM
Clara cells	Clara cell secretory protein (CCSP)	Anti-CCSP, rabbit polyclonal serum, Proteintech (10490-1-AP)	1: 30,000	Normal rabbit serum
Type II pneumocytes	Surfactant protein C (SP-C)	Anti-SPC, rabbit polyclonal serum, Jeffrey Whitsett, WRAB-9337	1: 3000	Normal rabbit serum
	Dendritic cell-lysosomal associated membrane protein (DC-LAMP)	Anti-DC-LAMP, rat monoclonal, IgG, clone 1010E1.01, Dendritics (DDX0191)	1: 200	Normal rat IgG
Neuroepithelial cells	Synaptophysin	Anti-synaptophysin, rabbit monoclonal, clone SP11, IgG, Vector Laboratories (VP-RM09)	1: 100	Normal rabbit IgG
All respiratory epithelium	Pan-cytokeratin	Anti-pancytokeratin, mouse monoclonal, IgG_1, clone AE1/AE3, Dako (M3515)	1: 500	Normal mouse IgG_1
Proliferating cells	Ki67	Anti-Ki67, rabbit polyclonal serum, Abcam (ab15580)	1: 1500	Normal rabbit serum

Figure 1. Morphology of foetal bovine lung tissue in different stages of gestational development - haematoxylin and eosin staining. a-pseudoglandular stage at 84 DGA, b-pseudoglandular stage at 98 DGA, note increasing airway density and different developmental morphology between them, c-transition between pseudoglandular and canalicular at 126 DGA, note first airspaces at the distal ends of terminal bronchioles and the presence of greater numbers of cells within mesenchymal tissue surrounding conductive airways, d-canalicular stage at 168 DGA, note very high density of cells in mesenchymal tissue and widespread presence of canaliculi which will develop into alveolar ducts and alveoli, e-transition between canalicular and saccular stages at 182 DGA, note first sacculi that arise after primary septation of canalicular units, f-alveolar stage at 266 DGA, g-alveolar cell organization at 266 DGA, note dense organization of cells within alveolar septa.



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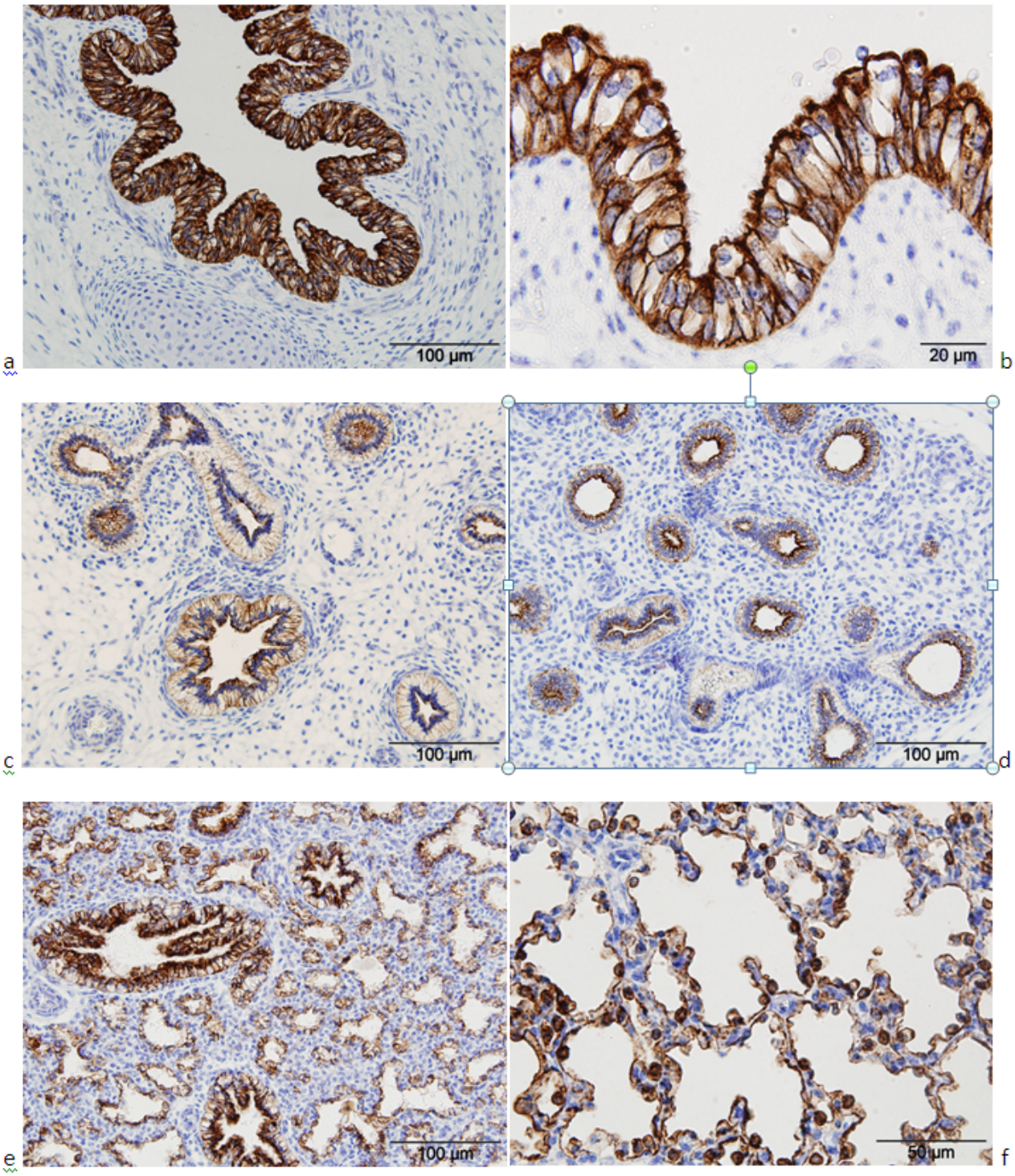

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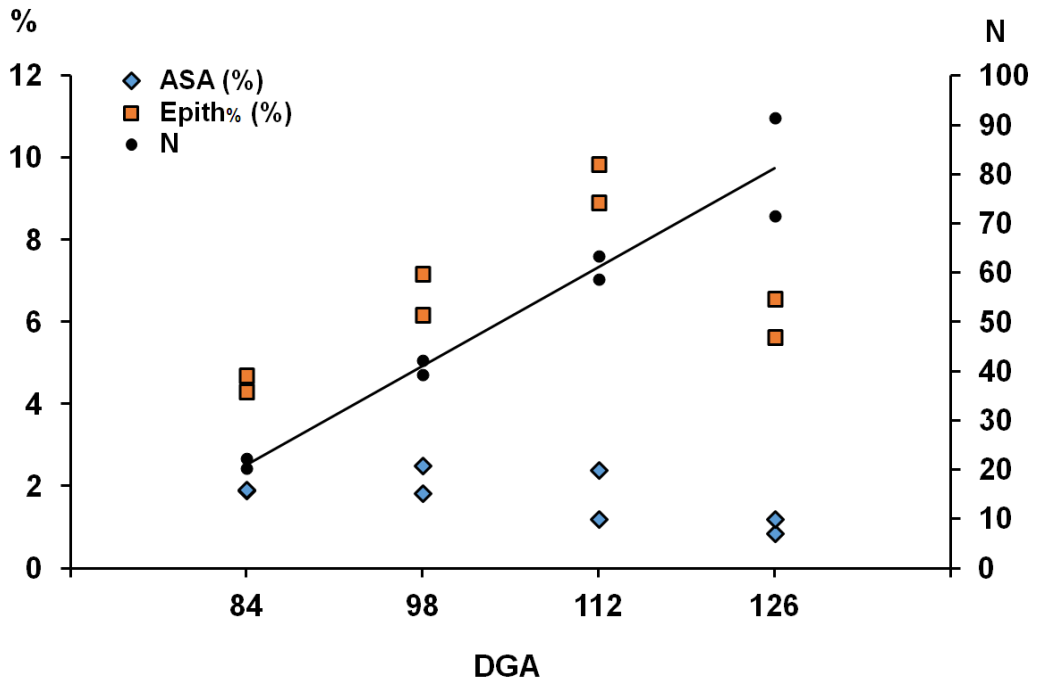

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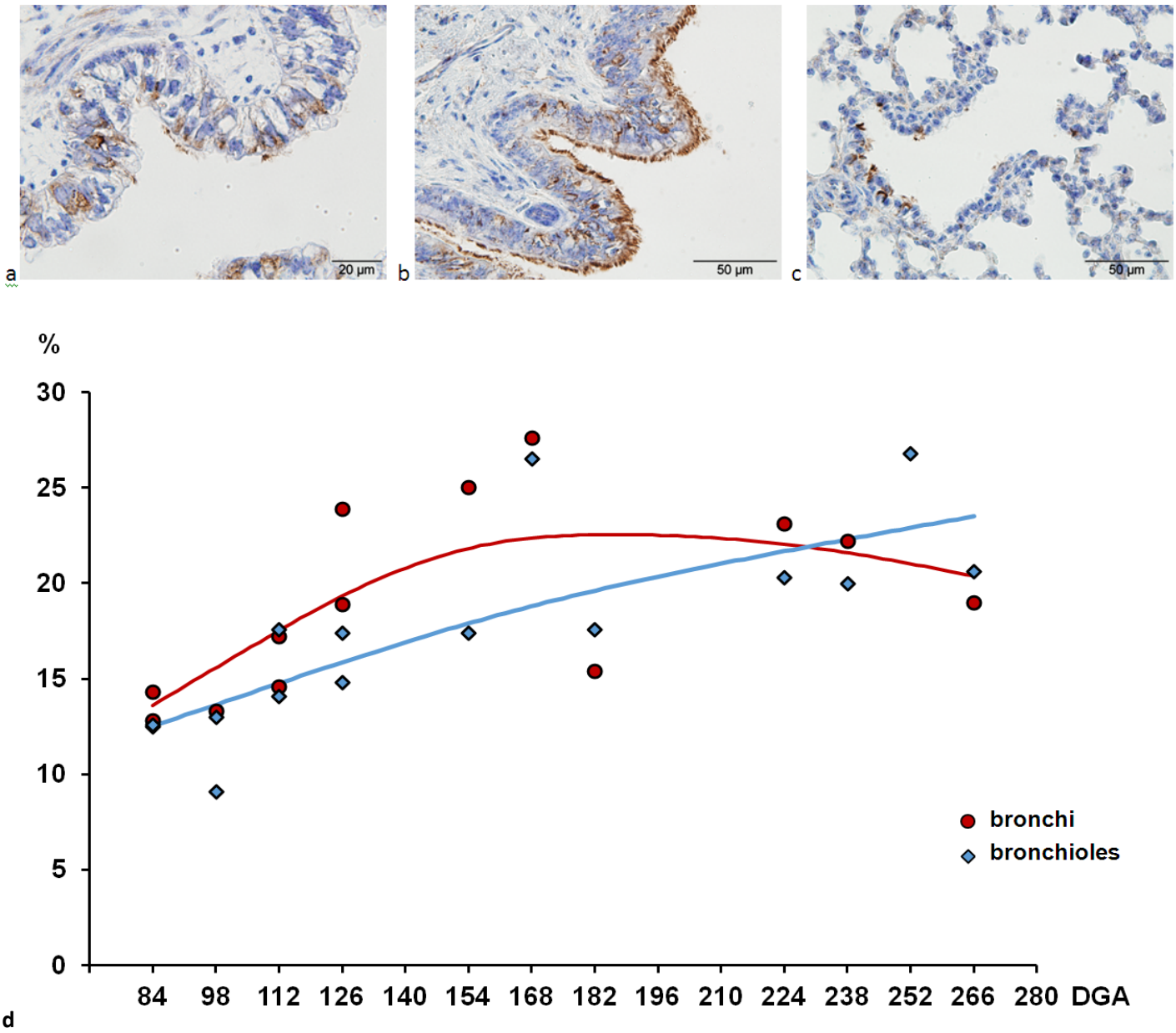

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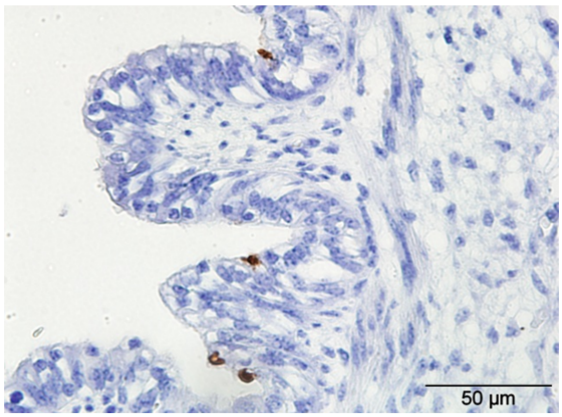
a



b

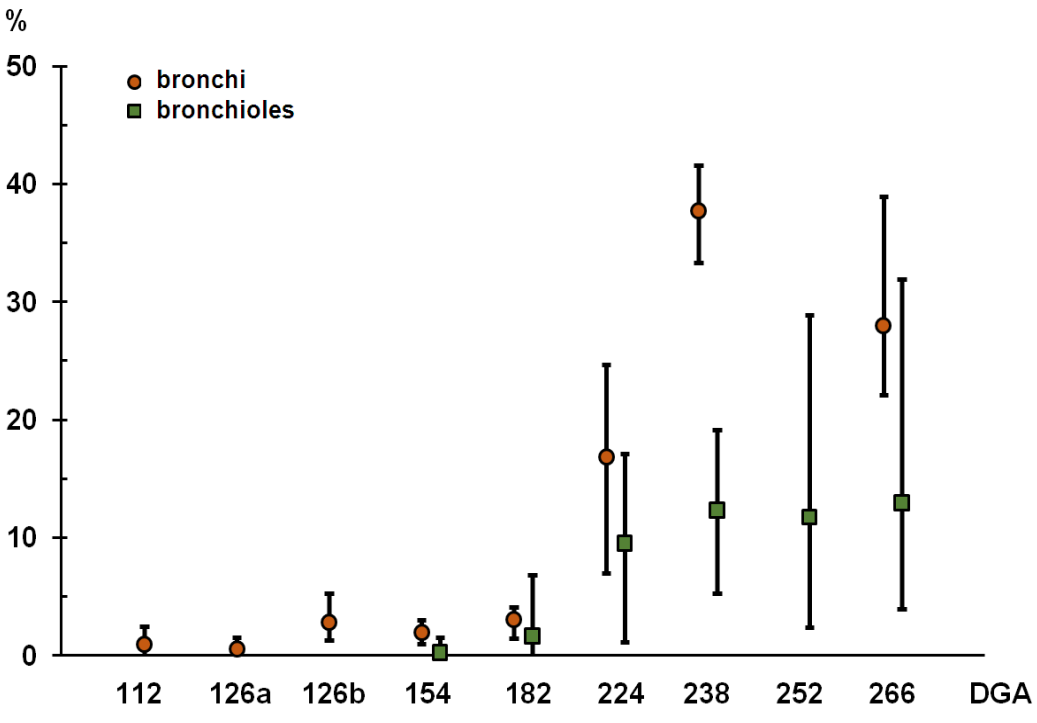
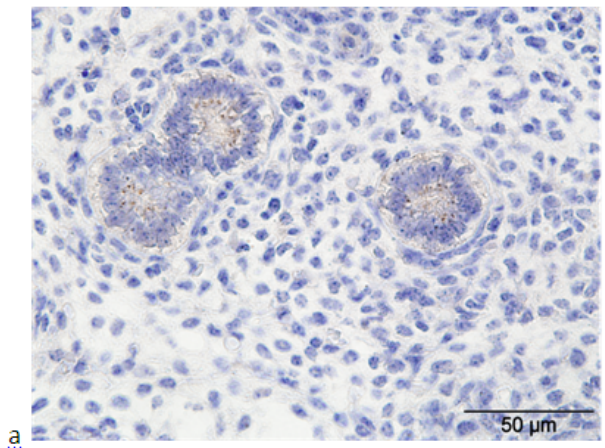


Figure 6. Type II pneumocytes as denoted by immunohistochemical labelling of surfactant protein C (SP-C) or dendritic cell-lysosomal associated membrane protein (DC-LAMP) (brown pigment). a- granules of SP-C protein in the lumina of developing airways at 112 DGA, note this marker of type II pneumocytes was widespread in epithelial cells of early developing airways, b- type II pneumocytes labelled by anti-SP-C antibody at 238 DGA, c- type II pneumocytes labelled by anti-DC-LAMP antibody at 266 DGA, note in the alveolar phase that both marker proteins of type II pneumocytes are present only in isolated single cells within the alveolar region, d- percentage of type II pneumocytes, as labelled by SP-C and DC-LAMP markers, within the alveolar region at the alveolar stage of bovine foetal lung development (DGA 224-266). The bars represent mean values and error bars depict minimum-maximum range.



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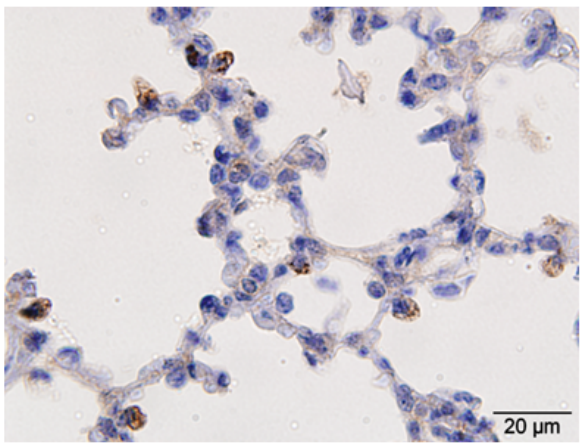

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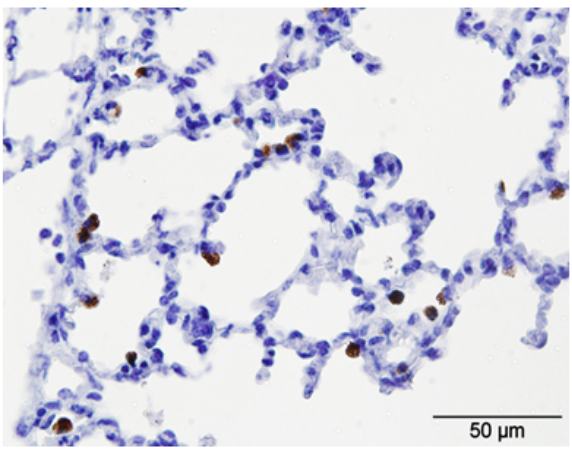
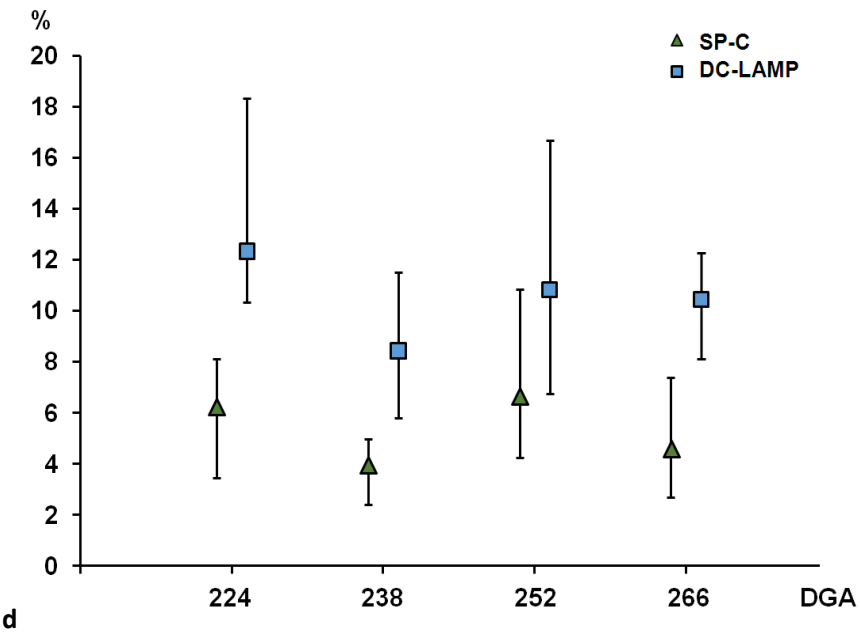
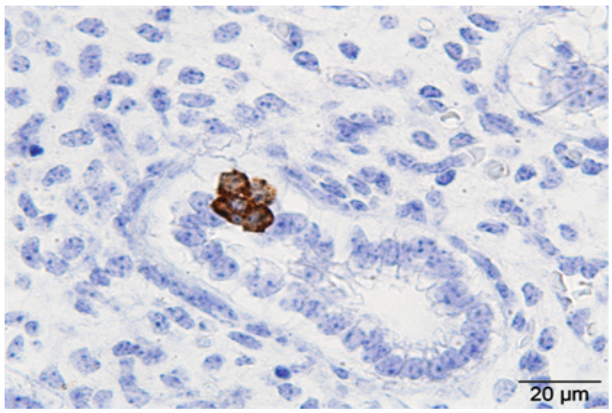


c

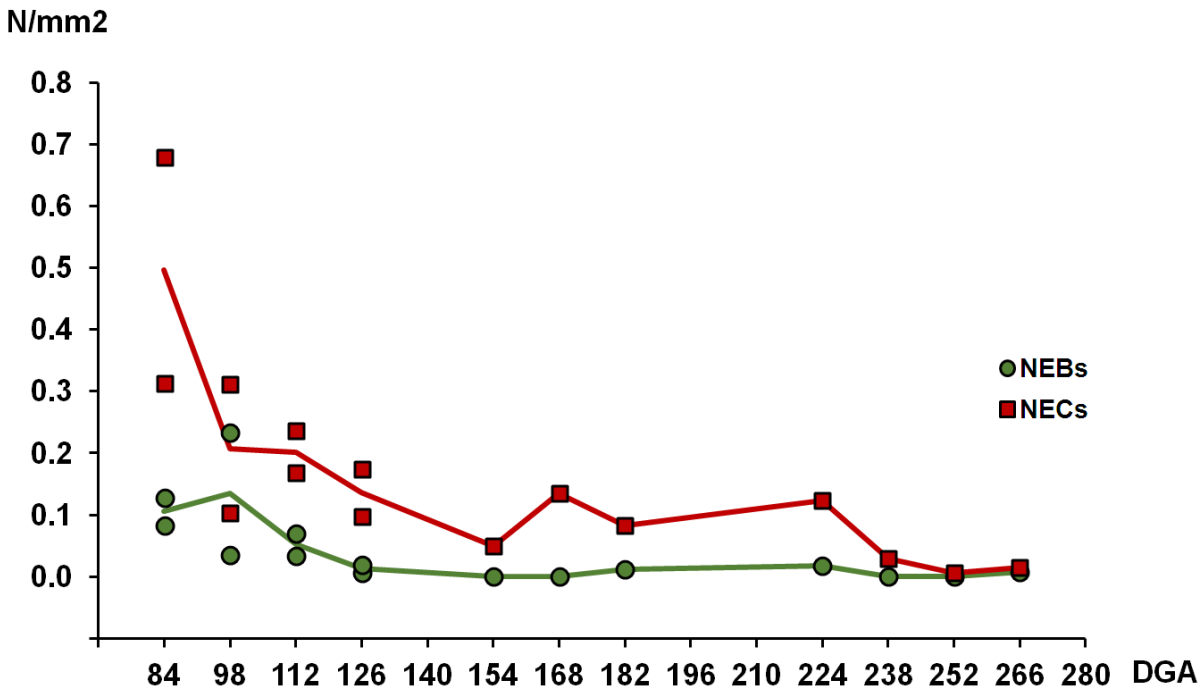


d

Figure 7. Neuroepithelial cells (NEC) and bodies (NEB) as denoted by immunohistochemical labelling of synaptophysin (brown pigment). a- neuroepithelial body (NEB) labelled within the epithelium of a developing distal airway at 126 DGA, b- mean number of NEC (red squares) and NEB (green circles) per mm^2 of total lung tissue in animals of different gestational ages. Trend lines were generated by joining mean values for numbers of NEBs/NECs in gestational time points examined.



a



b

Figure 8. Cell proliferation index (CPI), derived from immunohistochemical labelling of Ki-67, of bovine lung epithelium at different phases of foetal lung development. Data are depicted according to anatomical site, namely bronchi (●), bronchioles (■), distal developing airways (▲) and alveolar regions (♦). The figures represent means and vertical segments depict minimum-maximum range. a,b = different animals of the same gestational age. 98a DGA, 168 DGA and 252 DGA- no bronchi were present in the lung sections.

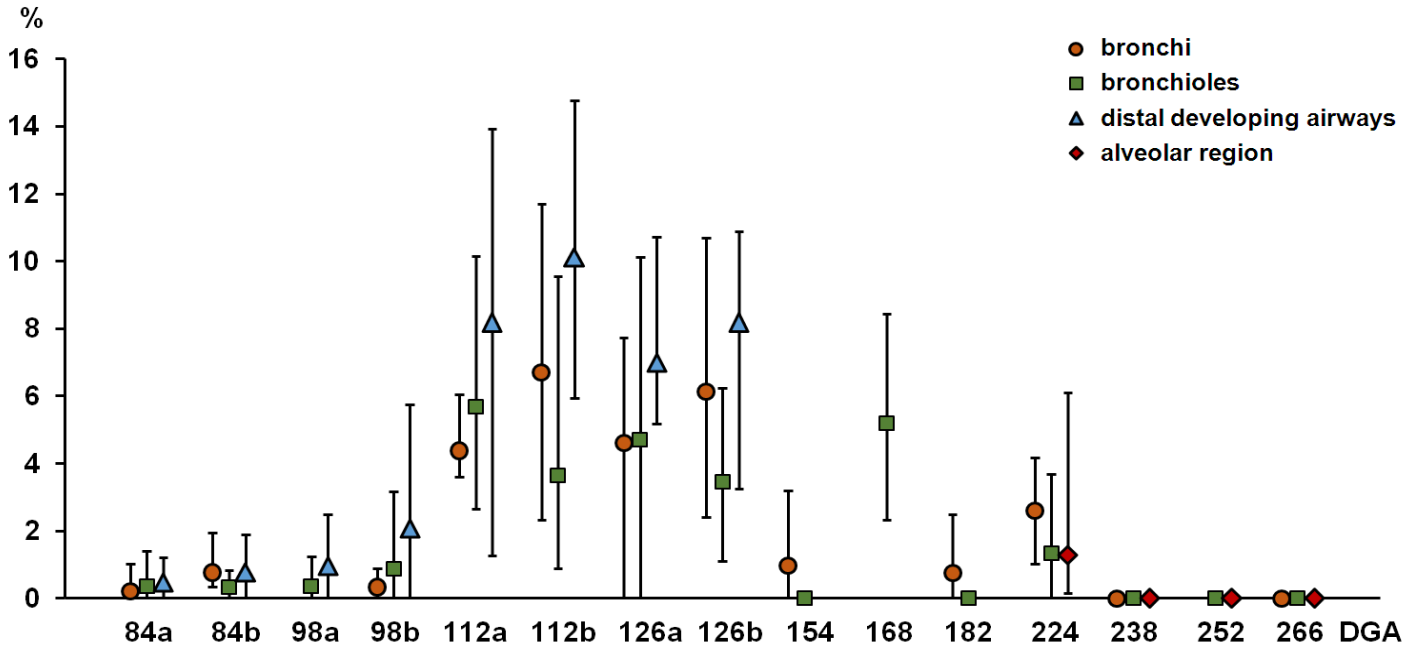


Figure 9. Diagrammatic representation of relative changes in different types of bovine pulmonary epithelial cells from day 84 until day 266 of gestation. Note, some changes are subjective (ciliation), some represent percentage within epithelium (type II pneumocytes, club cells) others are density (NEBs/NECs).