Branching morphogenesis in the avian lung: electron microscopic studies using cationic dyes

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SUMMARY

The developing chick lung was examined in the electron microscope for intimate cell contacts between epithelium and mesenchyme, discontinuities in the basal lamina and substructure of the basement membrane. Cell filopodia were seen which crossed the basal lamina from both the epithelial and the mesenchymal cells. Ruthenium red and tannic acid staining of the basal lamina of the chick lung showed it to be thin and sometimes discontinuous at the tips compared to the more substantial basal lamina in the interbud areas. The bilaminar distribution of particles seen with ruthenium red is similar to those seen in the cornea and lens. With tannic acid staining, filaments could be seen which crossed the lamina lucida and connected with the lamina densa. Spikes perpendicular to the basal lamina were sometimes seen with a periodicity of approximately 110 nm. Alcian blue staining revealed structure similar to that seen by ruthenium red staining in the salivary and mammary glands, although the interparticle spacing was closer.

Collagen was located in areas of morphogenetic stability, as has been seen by other investigators in different tissues. Collagen was coated with granules (probably proteoglycan) at periodic intervals when stained with ruthenium red. The fibrils were oriented circumferentially around the mesobronchus and were assumed to continue into the bud, but the fibres curve laterally at the middle of a bud. This orientation is opposite to that seen by another investigator in the mouse lung.

In general, the observations made in the avian lung are similar to those seen in branching mammalian tissue. It is likely, therefore, that the chick lung uses strategies in its morphogenesis that are similar to those that have been elucidated previously in developing mammalian organs.

INTRODUCTION

When an organ progresses from an anlage to a highly organized structure, interactions between the epithelial and mesenchymal tissues are necessary for proper development, as seen by the reciprocal dependence of the two tissue types on each other for their differentiation (Rudnick, 1933; Auerbach, 1960; Taderera, 1967).

The need for cell-to-cell contacts between epithelial and mesenchymal tissues has been well established in inductive events (Saxen et al. 1976) and several electron microscope studies have reported their presence in various mouse tissues (Cutler & Chaudrey, 1973; Coughlin, 1975; Bluemink, Van Maurik & Lawson,

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A second feature of branching morphogenesis is a basement membrane that becomes thinner or discontinuous at the tips of growing buds compared to interbud areas (Bernfield & Banerjee, 1972; Bernfield, Cohn & Banerjee, 1973; Coughlin, 1975; Lehtonen, 1975; see Bernfield, Bannerjee, Koda & Rapraeger, 1984, for review). Tannic acid and ruthenium red have been used to examine substructures of the basal lamina (the nonfibrillar layer of the basement membrane) in chick neural tube, notochord and cornea (Trelstad, Hayashi & Toole, 1974; Hay, 1978), chick lens (Hay & Meier, 1974), mouse salivary gland (Bernfield, Banerjee & Cohn, 1972; Cohn, Banerjee & Bernfield, 1977), and mouse mammary gland (Gordon & Bernfield, 1980).

Collagen is distributed preferentially in developmentally stable regions of various tissues (Kallman, unpublished observations; Grobstein & Cohen, 1965; Wessells, 1970; Mauger et al. 1982). Its arrangement in mouse lung is well ordered (Wessells, 1970), and comparisons of collagen distribution and orientation in the chick lung might relate to stabilizing morphology after initial morphogenetic events have occurred.

The chick lung is an advantageous system in which to study developmental events, because the buds emerge in a well-defined and characteristic sequence. Due to this conformation, a bud can be identified by the number of buds rostral to it, and its age determined by the number of buds caudal to it. A bud can be compared to homologous buds in other lungs at various ages, and can also be compared to buds in the same lung at different stages of development.

The present study was undertaken for the purpose of establishing the incidence of intimate cell contacts, discontinuities in the basement membrane at the tips of growing buds and the distribution of GAGs (glycosaminoglycans) and collagen in the developing chick lung. Ample evidence of close cell-to-cell contact was found. A correlation was made between patterns seen in the light microscope using lectins in a previous study and patterns seen in the electron microscope: a basal lamina that is thinner at the tips of growing buds than in the interbud area. The basal lamina contains an ordered substructure and collagen is preferentially distributed in the interbud area. Finally, the orientation of the collagen fibres is perpendicular to that previously found in the mouse lung.

**Materials and Methods**

**Electron microscopy**

White Leghorn eggs were incubated for 5–8 days and staged according to Hamburger & Hamilton (1951). The lungs were removed while the embryos were immersed in 3 % glutaraldehyde and 0-5 % paraformaldehyde in either a 0-11 M-Hepes or 0-08 M-cacodylate buffer at pH 7-4. The buffer used did not significantly alter the ultrastructural appearance.

Ten lungs were transferred to fresh fixative containing (1) 2 % tannic acid (Electron Microscopy Sciences) which was buffered to a final pH of 5-3 (Singley & Solursh, 1980) and postfixed in 1 % OsO4. Eleven lungs were placed in (2) 0-2 % ruthenium red (Sigma), pH 7-0 for 2 h and postfixed in 0-05 % ruthenium red and 1 % OsO4 in the dark, also for 2 h (Luft, 1971). Seven lungs were fixed for 2 h in (3) 1 % alcian blue, pH 6-35, followed by postfixation in 0-25 % alcian blue for 2 h (Behnke & Zelander, 1976). The mesothelial surface of lungs treated with these dyes was gently scraped prior to fixation to ensure the even penetration of ruthenium red and alcian
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blue. Seven lungs were processed in (4) the initial fixative without supplement and postfixed in 1% OsO$_4$ for 1 h (conventional fixation). The tissues were stained en bloc with 2% uranyl acetate in 0.05 M-sodium hydrogen maleate, pH 5.2, then dehydrated in ethanol, propylene oxide, and embedded in Epon Araldite.

Specimens were sectioned at 3 μm, collected on glass slides, stained with methylene blue and examined in the light microscope for areas of optimum interest and orientation, as well as for penetration of ruthenium red and alcian blue. Sections containing exact cross sections were selected, by taking the midpoint of the bud or the interbud area, in order to increase the accuracy of measurements of basal laminar thickness. Only the four earliest formed buds were examined so that comparisons could be made between buds of known age. A block was then glued to a chosen section with Devcon ‘5 minute’ Epoxy (Danvers, Mass.), then immersed in liquid nitrogen until the block snapped off the slide. The block was trimmed and sections were then cut, collected on slotted grids and stained with lead citrate. The material was viewed in a Hitachi HU 11E-1 electron microscope at 75 kV which had been calibrated with a carbon grating replica (Fullam).

Polarizing microscopy

For studies using the polarizing microscope, lungs were dissected out of embryos between stages 26 and 30 in a medium consisting of 1:1 horse serum (Irvine Scientific): Ham’s F-12 (Gibco). The lungs were placed in a 1% trypsin solution for 2–3 min at room temperature, after which the mesenchyme was removed using forceps and iridectomy knives.

RESULTS

General description

In a typical longitudinal (parasagittal) section, the mesobronchus is cut along its length, and the first few buds emerge from its dorsal surface in cranial–caudal sequence (Fig. 1). The area between two bronchial buds will be referred to as the interbud area. The most-distal extension of a bronchus will be referred to as a tip, and when a tip begins to branch the area between the two new branches will be called the new interbud area. The age of a lung will be referred to as the number of buds it has produced, e.g. a lung with 1 bud will be a 1-bud lung, a bud with 3 buds a 3-bud lung, etc.

Cell-to-cell contacts

Direct contacts are occasionally seen between epithelial and mesenchymal cells. They are located near the tips of newly emerging bronchi, rather than older bronchi or an interbud area, and consist of an extension from either cell type through discontinuities in the basal lamina. The tips of the extension come within 5–15 nm of the plasma membrane of the contacted cell. No specialized junctional structures were seen. In the interbud area, extensions from mesenchymal cells are frequently directed towards the epithelium, but the basement membrane is always intact in these cases, and therefore the cell layers are separated by at least its thickness (Fig. 2). In a lung at the 1-bud stage (stage 24), cell contacts are not seen in an area of the epithelial surface which 8 h later will give rise to the second bud. However, in this same specimen, the first bud has just begun to emerge, and along its basal lamina several mesenchymal filopodia come within 15–20 nm of the
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epithelial cells' plasma membranes. At the 5- to 8-bud stage (stage 28), epithelial cells at the tips of newly formed bronchi are seen with processes which extend through the basal lamina towards its mesenchyme. Sometimes the basal lamina remains over most of the surface of the cell process, but the tip is bare. In these cases, the epithelial–mesenchymal cell contact is closely apposed, and in one case is separated by only 5 nm (Fig. 3). At stage 30, intimate cell contacts are not seen in the four rostral buds.

Basal laminar discontinuities

The basal laminae of buds at different stages of development were compared for evidence of discontinuities at growing tips. As implied above, newly formed tips have basal laminae which are discontinuous and contain few structural details (Fig. 4). At the tips of buds formed 1 day earlier and in the interbud area, the basal lamina is distinct, continuous and is associated with copious fibrillar material on its mesenchymal surface (Fig. 5). New interbud basal laminae are similar to those of old interbud areas. Gaps in the basal laminae were not seen in areas expected to produce primary or secondary bud outgrowth.

Ultrastructure of basal lamina

In areas where the basal lamina is continuous, ultrastructural details can be discerned. Ruthenium red enhances the electron density of small particles that are present on either side of the diffusely staining lamina densa (Fig. 7) in older buds and interbud areas. The particles and the lamina densa are much less evident at the tips of new buds (Fig. 6). The particles are approximately 15 nm in diameter and are separated from adjacent particles in the same layer by 47 nm (±12 nm, n = 73 measurements, five lungs). The two layers of particles are approximately 60 nm apart. Periodic structures visualized by staining with alcian blue have an average separation of 58 nm (±15 nm, n = 52 measurements, three lungs) (Fig. 8). In tangential sections, the particles appear to be aligned vertically and horizontally in a tetragonal array (Fig. 9).

With tannic acid staining, the area of the basal lamina immediately adjacent to the plasma membrane (the lamina lucida) is transversed by thin fibres which

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Fig. 1. Semithin section of avian lung at 3-bud stage. The section is oriented dorsal side up, so that bud outgrowth is upward from the mesobronchus. ep, epithelium; mes, mesenchyme. ×265. Bar, 100 μm.

Fig. 2. The basement membrane (bm) of the interbud area is characterized by an extensive fibrillar layer and the basal laminar (bl) layer. The basal lamina is composed of the electron-dense lamina densa (ld) and the electron-lucent lamina lucida (ll). The mesenchyme (mes) is separated from the epithelium (ep) by the basement membrane. ×50000. Bar, 100 nm.

Fig. 3. A process from a 7-day epithelial cell (ep) extends through the basal lamina (bl) to contact a mesenchymal cell (mes). The basal lamina covers the surface of the process except for the tip, where the two cells are separated by only a 5 nm gap. ×82000. Bar, 100 nm.
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sometimes appear Y- or X-shaped (Fig. 10). The spacing between these filaments is 42 nm (±10 nm, n = 48 measurements, five lungs). The difference between the periodicities seen by ruthenium red and tannic acid is statistically significant (t-test, \( P < 0.01 \)), and therefore implies that different structures are bound by the two stains.

The distance between the centre of the lamina densa and the epithelial plasma membrane was compared between interbud areas (64 nm ± 13 nm, n = 13 measurements, four lungs) and tips (45 nm ± 16·17 nm, n = 31, four lungs) in ruthenium-red-stained tissue as a measure of basal laminar thickness. A significant difference between these two sets of measurements (\( P < 0.001 \)) can be detected, showing that the thickness of the basal lamina in an area of developmental stability (interbud area) is thicker than the basal lamina in an area of greater activity (the tips). Because the radius of curvature of the buds is +70 μm and that of the interbuds is −10 μm, the geometry conceivably could compress the basal lamina in the laminar plane to produce a thickening of approximately 0·6 %. The measured increase in thickness is almost 50 %.

Occasionally, tannic acid staining reveals fibrils with a length of approximately 18 nm normal to the surface of the basal lamina (Fig. 11). When two or three appear close together, their spacing is about 100 nm. Faint indications of similar structures can also be seen in routinely fixed tissue.

The basal surface of the epithelium is relatively smooth in the interbud area and tips (Figs 12, 13). Cytoplasmic filaments subjacent to the surface are usually aligned with respect to each other, but not with respect to an overall morphological feature, e.g. the axis of the bronchus, etc.

Localized electron-dense structures were seen on one mesenchymal cell close to the basal lamina in a lung stained with alcian blue. Electron-dense plaques are not apparent on cell membranes stained with ruthenium red. This may be due to understaining with ruthenium red, although electron-dense granules are present in the nearby extracellular spaces. Thin (3 nm) filaments are delineated in the extracellular spaces with ruthenium red, along which electron-dense granules, 10−30 nm in diameter, are positioned. Ruthenium red granules, 10 nm in diameter, also attach to collagen fibres (Fig. 15, inset).

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Fig. 4. The basal lamina at the tip of a newly emerged bud at 7 days is discontinuous in the areas marked by the arrows. Immediately above the basal lamina lies a mesenchymal cell. Tannic acid-fixed tissue. ×94000. Bar, 100 nm.

Fig. 5. The basal lamina at the interbud area of a 7-day lung is continuous, and is traversed by fine filaments across the lamina lucida. Above the basal lamina is fibrillar material. Tannic acid-fixed tissue. bl, basal lamina; ep, epithelium; mes, mesenchyme. ×94000. Bar, 100 nm.

Figs 6, 7. Comparison of tip and interbud area of ruthenium red-fixed tissue. bl, basal lamina. ×94000. Bar, 100 nm.

Fig. 6. A few large particles cover the tip. No regular substructure is seen.

Fig. 7. The basal lamina of the interbud area includes a diffusely staining band in the region of the lamina densa bordered on both sides by tiny granules (marked by small arrows).
Distribution and orientation of collagen fibres

Collagen fibres with an interperiod spacing of 55 nm are commonly associated with the mesenchymal surface of the basal lamina. They are more abundant in the interbud area than at the tips, and the striated fibres constitute a major portion of the fibrillar material that occurs at the epithelial–mesenchymal junction. The diameter of individual collagen fibres increases significantly from 18 nm (±4·6 nm, n = 20, five lungs) in 7-day lungs (stage 28) to 28 nm (±9·0 nm, n = 20, five lungs) one day later (P < 0·001). This pattern is seen even at new interbud areas. In two instances near tips of bronchi, collagen fibres closely associated with each other were observed to be in alignment with respect to interperiod spacing (Fig. 14). A third grouping was found out of alignment, revealing that collagen alignment is only an occasional feature of the chick lung.

While collagen fibres are not always aligned with each other, they are found to be roughly oriented with respect to overall tissue morphology. Thus, between bronchi, the fibres course through the saddle-like depression between the buds in a direction concentric with the circumference of the cylindrical mesobronchus (Fig. 15). A section grazing the lateral surface of the interbud mesobronchus shows the fibres to continue around the side of the structure (Fig. 16).

At the tips, although fewer fibres are found, they appear to be oriented with respect to the bud. The collagen at a 7-day unbranched tip is oriented medio-laterally with respect to the mesobronchial axis (Fig. 17). A grazing section of a branched tip shows the collagen fibres parallel to the groove separating the new buds on either side (data not shown). In the same section, but lateral to the branching tip, the basement membrane shows little evidence of collagen, demonstrating that the fibres are not concentric around the axis of the growing tip in a manner analogous to collagen around the mesobronchus. Mesenchymal cells do not demonstrate any preferred orientation relative to the collagen fibres.

Polarizing microscopy

Polarizing microscopy further demonstrates the orientation of collagen fibres. When a 3-bud lung is viewed through crossed polarizers, the presence of birefringence is detected by incomplete extinction of light. The compensator is rotated...
to a position where the specimen is seen in maximum contrast. As the specimen is rotated in a plane perpendicular to the microscope axis, the patterns of light and dark areas on the specimen also rotate, indicating that the light areas are not due to light scattered by the specimen. In Figs 18, 19, the specimen is viewed through crossed polarizers at two different angles, 90° apart, while the polarizers remain in a fixed position. The diagrams accompanying the photographs represent an idealized rendition of the positions of extinction. In the first photograph, a dark area appears at the tip and stalk of the bud and the lateral portions of the mesobronchus. When the lung is rotated 90°, these areas are now light. The anterior and posterior surfaces of the bud are bright in the first circumstance and dark in the second. This is consistent with a radial distribution of collagen around the tips, when viewed laterally, and a circumferential distribution around the mesobronchus. The distal extension of the mesobronchus also shows the same patterns of birefringence and, therefore, a radial distribution of collagen about the tip.

**DISCUSSION**

The bronchial basement membrane regions of 7- and 8-day-old chick lungs were examined by electron microscopy using tannic acid, ruthenium red and alcian blue to preserve GAGs and increase their electron density (Luft, 1971; Behnke & Zelander, 1976; Maupin & Pollard, 1983; Simionescu & Simionescu, 1975). It has been demonstrated recently that tannic acid stains HA (Singley & Solursh, 1980), and was employed in this study for that purpose.

**Cell-to-cell contact**

The importance of epithelial–mesenchymal interactions is well established (Grobstein, 1954, 1967; Cunha, 1976; Wessells, 1977; Goldin, 1980; Bernfield et al. 1984), yet the detailed mechanisms of these interactions are not known. In the present study, epithelial cells extended cell processes through the basal lamina at the tips of bronchi and approached mesenchymal cells within 5 nm. Specialized junctional complexes were not seen in the present study and dye-coupling techniques have not discovered any evidence of gap junctions in mammalian lungs in

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Figs 12, 13. The basal laminae of tips and interbud area stained with tannic acid. In both cases, the basal laminae are relatively smooth. ep, epithelium. ×11,600. Bar, 1 μm.

Fig. 12. The tip of a bud.

Fig. 13. The interbud area. Slight convolutions are seen.

Fig. 14. Collagen fibrils are aligned in lateral register (arrows). Tannic-acid-fixed tissue. This arrangement was found in the new interbud area of a branching bud. ×94,000. Bar, 100 nm.

Fig. 15. Section demonstrating the alignment of collagen in known areas of the basement membrane. Ruthenium-red-stained collagen sectioned in the plane indicated by the diagram: the interbud area. Collagen fibres course through the saddle in a direction concentric with the mesobronchial cylinder. ×51,000, inset ×190,000. Bar, 100 nm.
epithelial–mesenchymal interactions (Ryan et al. 1984). Mesenchymal cell processes contacted the epithelium through gaps in the basal lamina at a nascent tip. Intimate cell contacts were not seen prior to primary bud formation. They were also not seen prior to branching at the tips of six stage-30 lungs.
Intimate cell contacts have been seen in other organs undergoing branching morphogenesis: the mouse lung (Bluemink et al. 1976), mouse salivary gland (Cutler & Chaudrey, 1973; Coughlin, 1975) and mouse kidney during induction of the mesenchyme by the ureteric bud (Lehtonen, 1975). In mouse lung and submandibular gland (Bluemink et al. 1976), cell contacts formed after morphogenesis and were not seen until branching was well under way. Thus it seems that, in these mouse tissues, cell contacts are not needed to initiate branching, but are necessary for continued differentiation.

**Basal laminar folding**

The basement membrane of the chick lung is relatively smooth compared to the highly folded structures seen in the clefts of salivary glands (Bernfield & Wessells, 1970; Spooner & Wessells, 1972). In salivary gland, the rapid formation of clefts is considered a likely cause of the folds. In addition, the radius of curvature at salivary clefts is much smaller than at lung branch points; hence the lack of folding in lung basal laminae is not surprising.

**Discontinuities in the basal lamina**

The thinning of the basal lamina seen at growing tips has also been seen in mammalian tissues (Coughlin, 1975; Lehtonen, 1975). The distance between the lamina densa and the plasma membrane was significantly less at the tips than at the interbud area. It has been proposed that the function of discontinuities of the basal lamina is to allow contact between epithelium and mesenchyme (Bluemink et al. 1976; Goldin, 1980). Although the function of the contacts is not known, the close interaction between epithelial and mesenchymal tissues is necessary for morphogenesis to take place. In the present study, gaps in the basal lamina in areas expected to produce primary or secondary bud outgrowth were not seen, but if the time in which basal laminar discontinuity precedes bud outgrowth is short, e.g. 1 h, one would need to examine a much larger number of lungs in order to detect this event.

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Fig. 16. Section demonstrating the alignment of collagen in known areas of the basement membrane. Tannic acid-stained collagen sectioned on the lateral surface of the interbud area as indicated by the diagram. Mesenchymal cells show no particular orientation. ×11 600. Bar, 1 μm.

Fig. 17. Orientation of collagen at the tip of an unbranched bud in conventionally fixed tissue. Collagen fibres are aligned lateromedially with respect to the mesobronchial axis. ×50 000. Bar, 100 nm.

Figs 18, 19. Polarization microscopy of lungs. ×125. Bar in Fig. 18, 0-1 mm.

Fig. 18. Specimen viewed through crossed polarizers. The compensator has been adjusted so that its fast direction is vertical. An idealized version of the light and dark areas is depicted in the diagram. Arrow shows where alignment of collagen fibres is known through electron microscopy.

Fig. 19. The same specimen rotated through 90°.

Fig. 20. A schematic drawing of collagen fibres as interpreted by the results of polarizing microscopy.
Ultrastructure of basal lamina

Ruthenium red staining of the avian lung demonstrates the bilaminar arrangement of 15 nm particles along the lamina densa. The average interparticle distance of 47 nm is somewhat less than the interparticle spacing seen in similarly stained corneal tissue, 60 nm (Trelstad et al. 1974). Ruthenium red deposits were seen in the lamina lucida of the mouse salivary gland with a repeat of approximately 55 nm. In addition, 60–90 nm ellipsoids with a period of 100 nm were seen in the lamina densa (Cohn et al. 1977). While structures of this sort are not seen in the present study using ruthenium red, they are visualized with alcian blue staining.

In general, basal laminae of developing tissues seem to share common features: a lamina densa with a bilaminar distribution of GAG particles on either side of it, and anchoring filaments in the lamina lucida which connect the lamina densa and the plasma membrane of the epithelium. This is seen in developing tissues, branching or otherwise, chick and mouse tissue and is also seen in adult glomerular basement membrane (Farquhar, 1981).

Collagen distribution

In the present study, collagen was more abundant in the interbud areas of chick lung than at the tips. Collagen reduces the degradation of extracellular GAG in mammary tissue (David & Bernfield, 1979, 1981), and it probably serves the same purpose in the chick lung. The epithelial cytoskeleton may be responding to the presence of collagen via plasma-membrane-associated complexes with extracellular matrix receptors (Hay, 1985) to stabilize the epithelial configuration.

Collagen orientation

The collagen was oriented in a circumferential direction around the mesobronchus, as demonstrated by grazing sections of the basement membrane. Further elucidation of the collagen fibres was studied more easily by polarization microscopy (see Slayter, 1970).

In a specimen such as appears in Fig. 18, the dark areas represent the areas where collagen is oriented vertically. This interpretation is supported by the electron microscope observation that collagen is oriented vertically at the arrow. The dark areas vary continuously with rotation of the specimen, indicating that the collagen is arranged radially about the bud. The same radial orientation is observed around the distal extension of the mesobronchus, so that the overall arrangement of collagen fibres can be interpreted as in Fig. 20.

The organization of collagen fibres is exactly opposite to that seen in the mouse lung (Wessells, 1970), where a densely packed layer of collagen was oriented parallel to the tracheal axis. These differences may reflect different tensions in the developing mouse and chick lungs (Weiss, 1933; Stopak & Harris, 1982). Perhaps collagen orientation enhances growth along the long axis in a manner analogous to
that seen in the plant *Graptopetalum* (Green & Poethig, 1982). Finally, some aspect of ‘self-assembly’ might contribute to fibre alignment.

**CONCLUSIONS**

The developing chick lung was examined in the electron microscope to discover features of branching morphogenesis. These are: close cell contacts between epithelial and mesenchymal tissues, thin basal laminae at growing tips and thicker basal laminae at the interbud area. Detailed substructures of the basal lamina are similar to those seen in other developing tissues. A well-ordered lattice may be a general rule in basal laminae of developing organs, and perhaps of all basal laminae. Collagen orientation in the chick lung is perpendicular to that found in the mouse lung, but the reasons for this are not clear. In general, the chick lung uses strategies in its morphogenesis that are similar to those which have been elucidated previously in developing mammalian organs.

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**REFERENCES**


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