Stage specificity in the mesenchyme requirement of rodent lung epithelium \textit{in vitro}: a matter of growth control?

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**SUMMARY**

Epithelia from lung rudiments in which secondary bronchial buds are already established (14th and 13th gestational day for rat and mouse respectively) are able to undergo branching morphogenesis and cytodifferentiation in submandibular mesenchyme \textit{in vitro}, whereas lung epithelium from one day younger foetuses rarely gives a morphogenetic response to submandibular mesenchyme and usually differentiates into primary (non-budding) bronchial epithelium.

The failure of 13-day rat lung epithelium to respond to submandibular mesenchyme can be prevented by peeling off the submandibular mesenchyme from the lung epithelium after $2\frac{1}{2}$ days culture and replacing the same mesenchyme, or renewing it with fresh salivary mesenchyme \textit{ex vivo}. Changes in the epithelial contour are visible by 10 h and buds form within 24 h; this is followed by branching morphogenesis in more than 66% of the samples.

The number of cells in S-phase in the epithelium is doubled within 3 to 5 h after the operation and the number of mitotic cells (colchicine block) is increased during an 11 to 19 h period after the operation. Substituting stomach mesenchyme for submandibular mesenchyme after the operation failed to elicit morphogenesis or an increase in the number of S-phase cells in the epithelium.

The proportion of epithelial cells in S-phase in unoperated recombinates does not differ from the proportion in the primary bronchial epithelium (non-budding) of homotypic lung recombinates, whereas the proportion of S-phase cells in operated recombinates approaches that found in the buds of homotypic lung recombinates. The distribution of S-phase cells in visibly responding recombinates 15 to 17 h after operation shows the same heterogeneity as in homotypic lung recombinates, newly formed buds having twice as many cells labelled with $[^{3}H]thymidine$ as the non-budding area.

Cell cycle parameters of intact rat lung growing \textit{in vitro} were estimated using the labelled mitoses method. Primary bronchial epithelium and bronchial buds both had a total cell cycle time of about 13 h and an S-phase of about 10 h. The growth fraction was 0.54 in the primary bronchus and 0.95 in the buds. It is suggested that, also in the recombinates, differences in the proportion of S-phase cells at any one time in morphogenetically active and inactive areas of the epithelium are due to differences in the growth fraction.

It is concluded that an early event in the morphogenetic response of lung epithelium to submandibular mesenchyme after removing and restoring the mesenchyme is an increase in the size of the population of dividing cells and it is suggested that a high proportion of dividing cells in an epithelial population is a prerequisite for further interaction of epithelium and mesenchyme leading to branching morphogenesis.

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INTRODUCTION

Epithelia from organs that expand by branching, such as lung, salivary glands, the ureteric bud of the metanephric kidney, and mammary gland, have a fairly specific requirement for their own mesenchyme in vitro, with a limited interchangeability of mesenchymes between organs. When the epithelium is able to develop in foreign mesenchyme, the branching pattern is influenced and may be dictated by the mesenchyme, but the epithelium differentiates according to its origin: mammary gland epithelium branches in a salivary pattern in salivary mesenchyme (Kratochwil, 1969; Sakakura, Nishizuka & Dawe, 1976) but eventually produces milk proteins (Sakakura et al., 1976); analogous results have been found by interchanging mesenchymes between parotid and submandibular glands (Lawson, 1972; Ball, 1974). Salivary epithelium is able to cytodifferentiate in the absence of mesenchyme, and in the absence of continued morphogenesis, but only if the epithelium is isolated after branching has been initiated (Cutler, 1980); and Cutler has suggested that there is an obligatory link between early morphogenetic events and cytodifferentiation.

The lung primordium initiates in vitro, even from whole guts taken from 2-somite embryos (Spooner & Wessells, 1970) and so should be a suitable system for analysing tissue interactions from the earliest stages. Previous work had shown that, although lung epithelium may be viable in foreign mesenchyme (Dameron, 1961; Alescio & Cassini, 1962; Taderera, 1967; Spooner & Wessells, 1970; Alescio & Dani, 1971; Ball, 1974; Dürnberger & Kratochwil, 1980) it has a specific requirement for bronchial mesenchyme in order to undergo branching morphogenesis. Only Taderera (1967) reported a limited branching response to salivary mesenchyme by mouse lung epithelium at a stage when secondary bronchial buds were well established; and young lung primordia occasionally respond to salivary mesenchyme (Spoon and Wessells, 1970). In contrast, the initial outpocketing of lung primordia from gut endoderm can take place in the presence of salivary mesenchyme (Spoon and Wessells, 1970) and both salivary and stomach mesenchyme can induce supernumerary buds from tracheal epithelium (Wessells, 1970), but neither primordia nor tracheal buds develop further except in the presence of bronchial mesenchyme.

A mechanism of branching morphogenesis involving the proteoglycan interface material between mesenchyme and epithelium and the contraction of epithelial microfilaments has been proposed for salivary gland (Bernfield & Wessells, 1970; Bernfield, Banerjee & Cohn, 1972; Spooner & Wessells, 1972; Banerjee, Cohn & Bernfield, 1977; Bernfield, 1981; Bernfield & Banerjee, 1982). The following observations suggest that there are at least common elements in the branching mechanisms of different branching organs. Firstly, the distribution of interface acid glycosaminoglycan is similar in salivary gland, lung, ureteric bud, mammary gland and the urogenital tract (Bernfield & Banerjee, 1978; Cunha, 1976). Secondly, there is comparable sensitivity of some of these
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rudiments, compared with non-branching ones, to collagenase (Wessells & Cohen, 1968), and to interference with collagen synthesis (Alescio, 1973; Spooner & Faubion, 1980) and glycosaminoglycan synthesis (Thompson & Spooner, 1982). Thirdly, salivary epithelium is able to continue branching in lung mesenchyme (Ball, 1974; Lawson, 1972, 1974), ureteric bud can develop in salivary and in lung mesenchyme (Lawson, unpublished), and mammary epithelium can develop in salivary mesenchyme (Kratochwil, 1969; Sakakura et al., 1976). The present work was undertaken to clarify the apparent failure of lung epithelium to undergo branching morphogenesis in other than bronchial mesenchyme.

Lung epithelium, from stages before and after secondary bronchial bud initiation, has been recombined with its own and with salivary mesenchyme.

MATERIALS AND METHODS

Animals

Wistar rats and Swiss mice were used. The morning on which vaginal sperm were found (rats) or a copulation plug was present (mice) was defined as gestational day 1.

Tissues and culture method

Lungs were obtained from 14-day and 13-day rat foetuses and from 13-day and 12-day mouse foetuses. Submandibular gland rudiments were taken from 16-day rat and 14-day mouse foetuses. The stage of development of these rudiments at dissection is illustrated in Lawson (1974). Epithelia were eased out of the mesenchyme with tungsten needles after treatment with 0.1% crystalline trypsin (Worthington) and 0.27% pancreatin (Difco) in Tyrode's solution or Dulbecco's PBS for 5 min at 37°C. The enzymes were inactivated with 50% foetal calf serum. The recombinates, composed of epithelium from one and mesenchyme from two rudiments, were supported by a thin film of agar. One percent agar was allowed to gel across a hole cut in a strip of cellulose acetate net. The strip was then lain on a clot of cock plasma and chick embryo extract (2:1) (Lawson, 1972). In experiments in which [3H]thymidine incorporation was followed, the tissues were supported by a Nuclepore filter (0.1 μm nominal pore diameter) over a medium of cock serum (prepared from plasma) and chick embryo extract. The cultures were incubated at 37°C in a humidified mixture of 5% CO2 in air.

Bud number and epithelial expansion were estimated from photographs of the living material, taken through a Zeiss 16 mm Luminar objective with open diaphragm.

Histology

Cultures were fixed for 8 h in Helly's fluid and embedded in Histowax. Sections 5 μm thick, with and without amylase treatment, were stained with periodic acid-Schiff (PAS), alcian blue and Mayer's haemalum.
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[3H]thymidine incorporation

a) Cells in S-phase. Cultures were labelled with [3H]thymidine (5 μCi/ml, 48 Ci/mmol) for 2 h, rinsed three times with 10% foetal calf serum (FCS), fixed for 30 min in Carnoy's fluid at 0°C, dehydrated, and embedded in Histowax.

b) Cell cycle parameters. 14-day rat lungs were pulsed after 24 h culture with 5 μCi [3H]thymidine/ml for 30 min, rinsed with Dulbecco's PBS containing 3 μM-thymidine and 30% medium, cultured further on medium containing 3 μM-thymidine, and fixed in Carnoy's fluid.

Autoradiography

Sections 3 μm thick were coated with liquid nuclear track emulsion (Kodak NTB2) (Kopriwa & Leblond, 1962) and exposed at 4°C, 0% humidity for 4 days (cells in S-phase) or 8 days (cell cycle parameters), developed in Dektol 1:1 for 2 min at 19°C, fixed in Kodak F5, and stained with PAS-Mayer's haemalum. Periodic acid oxidation was performed before coating with emulsion, staining with Schiff's solution and haemalum after autoradiographic processing (Kopriwa & Huckins, 1972). Amylase treatment of the sections showed that the presence of glycogen does not influence the grain density (W. Sluiter, personal communication). Sections of identically processed, unlabelled cultures showed no indication of positive chemography or negative chemography after controlled fogging.

Microscopical analysis

a) Colchicine Index (CI). The proportion of cells with blocked mitoses was counted in every fourth section of areas containing epithelium. Cultures had been treated with 0.25 μM-colchicine for 8 h before fixation.

b) Cells in S-phase. Cells with four or more grains/nucleus when viewed through a ×40 objective with bright field were considered labelled. The proportion of labelled nuclei was estimated on cross sections of the epithelium perpendicular to the basement membrane. For each sample a total of ca. 1500 cells was counted in three to five sections.

c) Cell cycle parameters. Labelled mitoses were counted through a ×100 Zeiss Epiplan objective using a combination of bright and dark field. At least 150 mitoses both in the primary bronchi and in the buds (secondary bronchi) were counted. Since the grain density in the epithelium was relatively low, background correction was made on the basis of the mean grain frequency distribution over epithelial mitoses in explants fixed directly after labelling and after 30 min chase. The labelling index of the epithelium (LI = fraction of labelled cells) was estimated on the same explants fixed directly after labelling and after 30 min.
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chase; correction for background was made with the grain frequency distribution over epithelial cells of unlabelled explants. In practice, it was only necessary to correct 2-grain mitotic cells and 1-grain interphase cells.

Statistical analysis

Incidence of response was tested with the \( \chi^2 \) test. Analyses of variance were performed on bud number after transformation to \( \log_{10} \), on other data without transformation. The t-test was used where applicable.

Abbreviations

Recombinates are abbreviated as follows: LL: lung epithelium with lung mesenchyme; LS: lung epithelium with submandibular mesenchyme; L13S: 13-day rat lung epithelium with rat submandibular mesenchyme; L14S: 14-day rat lung epithelium with rat submandibular mesenchyme.

RESULTS

Stage specificity in mesenchyme requirement

Left lung epithelia, in which one or more bronchial buds were established (14-day rat, 13-day mouse) underwent branching morphogenesis in salivary mesenchyme, whereas epithelia from sac-like primordia (13-day rat) or from primordia in which shape changes heralding bronchial bud formation were present (12-day mouse) generally failed to respond, although the homotypic recombinates developed normally (Fig. 1). The results were more clear cut in the rat material: a significant proportion of the mouse epithelia from both ages (23% and 37%) formed extensions into the mesenchyme which failed to branch.

The epithelium in all homotypic recombinates (LL) expanded rapidly: branching morphogenesis was reinitiated after 24h in recombinates from older foetuses, but was not evident in the recombinates from 13-day rat epithelia until the end of the second or during the third day. The rate of epithelial expansion in all LS recombinates (measured planimetrically; data not shown) was about half that in the homotypic recombinates. However, there was no delay in reinitiation of morphogenesis in the responding LS recombinates from older foetuses, compared with the LL controls. Unlike the homotypic recombinates, in which branching buds were initiated at several places, responding LS recombinates usually formed only one or two extensions into the mesenchyme that continued to branch, and then in a salivary fashion (Lawson, in preparation).

The epithelia of LS recombinates showing a positive response had developed after 8 days into two histologically distinct areas: a) ciliated, columnar epithelium with or without glycogen and with a scattering of cells containing non-glycogen PAS-positive secretory material (Fig. 2B). This epithelium is indistinguishable from the primary bronchial epithelium in intact lung and LL
recombinates (Fig. 2D, E); b) cuboidal and columnar epithelium containing glycogen (Fig. 2C). This epithelium of the buds and branches resembles the epithelium that develops in the future respiratory area of the lung and in homotypic recombinates (Fig. 2F), except that the lumina are more expanded in the heterotypic recombinates (Fig. 2A, D). The difference may be due to the absence of peristalsis in the recombinates with salivary mesenchyme. The epithelium of non-responding LS recombinates differentiated into primary bronchus-like epithelium only (Fig. 3A, B).

All further experiments were done with rat material because the difference in response between older and younger stages was more clear cut than with the mouse material.

**Epithelial size**

The slower epithelial expansion in LS recombinates and the delay in onset of branching morphogenesis in homotypic recombinates of young primordia (13-day rat) suggested that 13-day rat lung epithelium might not be able to reach a critical size necessary for new bud formation before losing its ability to respond

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**Fig. 1.** Frequency of morphogenetic response (branched buds) of lung epithelium from rat and mouse foetuses at different developmental stages. The epithelium was recombined with its own or with salivary mesenchyme. Open columns – LL controls; hatched columns – LS; stippled columns – LS showing doubtful response (one or more buds, no branching).

**Fig. 2.** Recombinates of 14-day rat lung epithelium with salivary mesenchyme (A–C) or with lung mesenchyme (D–F), after 8 days *in vitro*. PAS-alcian blue–haemalum. (B), (E): ciliated, columnar epithelium containing PAS +ve secretion (arrows). Amylase treated. (C), (F): cuboidal epithelium of branched portion. Dark staining is glycogen. Scale bar (A) and (D) = 100 μm. Scale bar (B), (C), (E) and (F) = 20 μm.
Fig. 2
Fig. 3
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Fig. 4. Frequency of morphogenetic response of rat lung epithelium of different size and developmental stage. (A): whole left or right lung epithelium from 14-day rat foetuses. (B): distal end of lung epithelium from 14-day rat foetuses. (C): whole left or right lung epithelium from 13-day rat foetuses. Open columns – LL; hatched columns – LS; stippled columns – LS showing doubtful response (one or more buds, no branching).

to salivary mesenchyme. There was also the possibility that salivary mesenchyme had lost its ability to initiate branching morphogenesis before the epithelium had reached the critical size.

Whether initial epithelial size is a limiting factor of response in heterotypic recombination was tested by comparing the response for the distal tip of 14-day lung epithelium with that of whole 14-day epithelium and whole 13-day epithelium. That the distal tip had the same initial size as the 13-day primordium was checked planimetrically on photographs of the recombinates. The distal tip showed branching morphogenesis in 53 % of the samples, which was significantly more ($P < 0.01$) than the 6 % response of the 13-day epithelium and less ($P < 0.05$) than the 86 % response of the whole left lung epithelium (Fig. 4). Epithelial expansion of 14-day distal tips in salivary mesenchyme was faster than the expansion of whole 13-day epithelia in salivary mesenchyme: the area of the distal tip increased from $3 \times 10^4 \mu m^2$ to $11.6 \times 10^4 \mu m^2$ during the first 60 h compared with an increase by 13-day epithelia from $3.24 \times 10^4 \mu m^2$ to $6.32 \times 10^4 \mu m^2$ ($t = 3.44$, df $= 13$, $P < 0.01$).

Therefore, although small initial size of the lung epithelium reduces the

Fig. 3. Recombinates of 13-day rat lung epithelium with salivary mesenchyme, 8 days in vitro. (A), (B): control recombinates. (C–E): epithelium and mesenchyme separated after 64 h culture and reassociated. PAS-alcian blue-haemalum. (B), (E): PAS-positive secretion (arrows) in columnar epithelium. Amylase treated. (D): glycogen containing epithelium of branched portion. Scale bar (A) and (C) = 100 \mu m. Scale bar (B), (D) and (E) = 20 \mu m.
probability for branching morphogenesis in salivary mesenchyme, it does not fully account for the stage difference in response between 14-day and 13-day epithelia.

**Ageing of salivary mesenchyme**

The possibility that salivary mesenchyme loses the ability to initiate branching morphogenesis before 13-day rat lung epithelium is large enough to respond was tested by peeling off the mesenchyme from L₁₃S recombinates after 2½ days culture and renewing it with three fresh salivary mesenchymes from 16-day foetuses. Unoperated L₁₃S recombinates and recombinates in which the salivary mesenchyme was peeled off, cut into three pieces, and then replaced served as controls. No enzymes were used, and a thin layer, or patches, of mesenchyme cells remained adhering to the epithelium.

The results (Table 1) showed that both renewing the mesenchyme and replacing the same mesenchyme led to an increase in the incidence of branching morphogenesis. The incidence of response by left lung epithelium was slightly higher than that of right lung. When all available data were considered, the left lung epithelium showed a branching response in 70 % (N = 116) of the samples, the right lung epithelium in 53 % (N = 104) (P<0.05). However, the control L₁₃S recombinates showed branching morphogenesis in 21 % (N = 42) when left lung epithelium was used and only in 5 % (N = 40) of the control recombinates with right lung epithelium (0.05 <P<0.1).

Table 1. *Incidence of response of left and right lung epithelia from 13-day rat foetuses, recombined with salivary mesenchyme*

A: control.
B: mesenchyme removed and replaced after 64 h culture.
C: mesenchyme removed after 64 h culture and renewed with fresh salivary mesenchyme from 16-day foetuses.
-: no budding.
±: one or more buds, no branching.
+: branching morphogenesis.

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The response was usually visible within 24 h after the operation as a single, sometimes more than one, bud (Fig. 6C) that then branched continuously (Fig. 5) in a salivary fashion (Fig. 6D, E).

Epithelial differentiation of these responding recombinates was less advanced at 8 days than the L14S recombinates (Fig. 3C, D, E).

It is concluded that mechanical removal and replacement of the same salivary mesenchyme after 2½ days culture is sufficient to allow a morphogenetic interaction between the epithelium from the 13-day rat lung primordium and salivary mesenchyme. Renewing the mesenchyme produces the same result, but is not a necessary condition for it.

Initial shape changes

The morphological changes occurring in the epithelium during the first 20 h after separating and reassociating epithelium and mesenchyme from 2½-day cultured L13S recombinates made with left lung epithelium were examined. Recombinates in which the separated epithelium was reassociated with precultured stomach mesenchyme from 13-day foetuses instead of with salivary mesenchyme,

Fig. 5. Response of 13-day rat left lung epithelium to salivary mesenchyme after replacement or renewal of the mesenchyme after 64 h culture. Solid circles – mesenchyme replaced; open circles – mesenchyme renewed with fresh salivary mesenchyme from 16-day foetuses; broken line – mesenchyme left undisturbed; dotted line – LL control. Vertical line represents 95 % confidence limits derived from the analysis of variance.
Fig. 6. Response of 13-day rat lung epithelium to salivary mesenchyme after separating and replacing the same mesenchyme in a 64 h-old recombinate. (A): before operation. (B): 10 h. (C): 18 h. (D): 48 h. (E): 75 h. (Blemishes on lower part of some photos are due to threads of cellulose acetate supporting the agar substratum.) Scale Bar = 200 μm.

were used as controls. This operational control mimics the spatial arrangement of operated recombinates, but does not stimulate morphogenesis of the epithelium. Although the contour of the epithelium in association with stomach mesenchyme was more irregular during the first 19 h after the operation than before in 19% of the samples (N = 95), these changes did not presage definitive
bud formation. Contour changes recognisable as early bud formation were present after 8–10 h (Fig. 6A, B) in 50% of L13S recombinates in which the mesenchyme had been removed and replaced (N = 32) and in 75% by 14 h (N = 36).

**Growth stimulus**

Planimetric measurements of epithelial expansion after operation of L13S recombinates at 2½ days (data not shown) suggested that epithelial growth was stimulated by the operation.

2½-day-old L13S recombinates, in which the mesenchyme had been peeled off and then put back, and unoperated L13S recombinates (controls) were treated with 0-25 μM-colchicine for 8 h, either 3 to 11 h, or 11 to 19 h after operation. The colchicine index (CI) of the epithelium was increased by 50% in the operated recombinates compared with the controls during the 11–19 h period, but was unaffected during 3 to 11 h after operation (Fig. 7). Removing and replacing the mesenchyme had no effect on the CI of the mesenchyme itself during either period.

The CI of the epithelium of operated recombinates during the 11–19 h period approached that of the epithelium of homotypic lung and salivary gland recombinates (Fig. 8), whereas peeling without replacing the mesenchyme led to a 40% drop in the CI of the epithelium compared with the unoperated controls.

The proportion of epithelial cells in S-phase was measured during three 2 h periods during the first 19 h after operation (Fig. 9). The proportion of nuclei labelled with [3H]thymidine was higher in the operated recombinates 3–5 h after operation (P < 0.01) (Fig. 10A–D), and at 15–17 h; the difference at 9–11 h was not statistically significant. The proportion of labelled nuclei in the epithelium of the controls was indistinguishable from that of the morphogenetically inactive

![Fig. 7](image-url)
Fig. 8. Colchicine index of epithelium in recombinates 11–19 h after removal and replacement of mesenchyme in 64 h-old L13S recombinates. Open column – L13S control; hatched column – salivary mesenchyme removed and replaced; stippled column – mesenchyme removed and not replaced; scattered hatching – LL control; circles – SS control. Vertical line represents 95% confidence limits derived from the analysis of variance.

Fig. 9. Percentage labelled nuclei in the epithelium after labelling for 2 h with $^{[3]H}$-thymidine at different periods after removing and replacing the mesenchyme of 64 h old L13S recombinates. Open columns – L13S controls (mesenchyme undisturbed); scattered hatching – L13S mesenchyme replaced with stomach mesenchyme; solid columns – L13S mesenchyme removed and replaced; broken hatching – LL, non-budding area; hatched columns – LL, buds. Vertical lines represent 95% confidence limits derived from the analysis of variance.

Fig. 10. Autoradiographs of recombinates of 13-day rat left lung epithelium with salivary or lung mesenchyme, labelled for 2 h with $^{[3]H}$-thymidine. (A), (B): L13S mesenchyme removed and replaced at 64 h; labelled 3 h after operation. High labelling index in epithelium. (B) is dark field of (A). (C), (D): L13S control: labelled at same time as (A), (B). Moderate labelling index in epithelium. (D) is dark field of (C). (E), (F): L13L control: labelled at same time as (A), (B). High labelling index in epithelium of buds (long arrows), moderate in established branch points and non-budding portion (short arrows). (F) is dark field of (E). (G), (H): L13S. Operated recombinate, labelled 15 h after operation. High labelling index in new buds (long arrows), low in non-budding area (short arrows). (H) is dark field of (G). Scale bars = 100 $\mu$m.
parts of the homotypic LL recombinates, whereas the proportion in lung epithelium after removing and replacing salivary mesenchyme approached that of the actively budding area of homotypic lung recombinates (Fig. 9). The epithelia of operated L13S recombinates showing a clear difference in the living cultures between budding and non-budding areas at 17 h also showed a heterogeneous distribution of labelled nuclei similar to the LL recombinates (Fig. 10E–H, Fig. 11).

Recombining the separated epithelium with precultured stomach mesenchyme, instead of replacing the salivary mesenchyme, did not lead to any enhancement of the number of labelled nuclei in the epithelium (Fig. 9).

It is concluded that removing and replacing salivary mesenchyme causes a rapid increase in the number of cells in S-phase in the epithelium, before morphogenetic shape changes are clearly recognizable; this increase is maintained in visibly extending buds.

Cell cycle parameters

Interpretation of the above results is ambiguous without some knowledge of the cell cycle parameters of lung epithelium. These were estimated on cultures of intact lungs, precultured for 24 h, from 14-day foetuses, using the labelled mitoses method. The results (Fig. 12) show that, within the limitations of the method, the total cell cycle time of dividing cells (T) in the primary bronchus and in the bronchial buds is the same (12.9 h) with an S-phase duration of 10 h, G2 + ½M of 2.6 h, leaving a G1 + ¼M of 0.3 h. The failure to find 100% labelled mitoses is thought to be due mainly to the use of 3 μm sections coupled with the relatively low grain density in the epithelium (Modak, Lever, Therwath & Uppuluri, 1973). Correction of the data on this assumption did not lead to a significant change in the estimates of T and S (12.7 and 9.9 respectively).

The theoretical labelling index (theoretical LI = S/T) is 0.775, which is the...
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Fig. 12. Percentage labelled mitoses in epithelium of 14-day rat lungs in vitro, at different times after the start of a 30-min pulse with $[^3]H$thymidine. Open circles – primary bronchus; solid circles – buds. The duration of the phases of the cell cycle are obtained from the 50% intercept as follows: Total cell cycle time ($T$) = $D-B$; $S = C-B$; $G_1 + G_2 + M = D-C$; $G_2 + iM = B-A$.

proportion of labelled cells expected after a short labelling time if all cells are cycling. The experimental labelling indices of primary bronchus and of bronchial buds were measured in duplicate after 0.5 h labelling and found to be 0.467, 0.367 in the bronchus and 0.704, 0.761 in the buds. These values yield a mean growth fraction (experimental LI/theoretical LI) of 0.54 for the bronchus and 0.95 for the buds.

In applying these data to the recombinates, it is assumed that the homotypic $L_{13L}$ recombinates after 2.5 days culture are analogous to intact 14-day lungs after 1 day in culture. The difference in the proportion of cells in S-phase in morphogenetically active and inactive areas of the recombinates is therefore not due to differences in cell cycle parameters, but to differences in the proportion of cycling and non-cycling cells: bronchial buds have double the number of cycling cells compared with the bronchus. By analogy, removing and replacing the salivary mesenchyme of $L_{13S}$ recombinates initiates DNA synthesis in the part of the epithelial population which had stopped multiplying.

**DISCUSSION**

The ability of lung epithelium to undergo branching morphogenesis in salivary mesenchyme is stage dependent: epithelia from lungs in which one or more lateral buds have been established (14-day rat, 13-day mouse) respond to salivary mesenchyme, although epithelial expansion is slower than in the homotypic recombinates. Epithelia from sac-like primordia (13-day rat) or from primordia in which lateral buds are not fully established (12-day mouse) fail to branch, a result for which the small initial size of the epithelium can be only
partially responsible. This suggests that the mesenchyme requirement for the development of the lateral buds that will become the secondary bronchi is more critical than that for establishing the initial lung primordia (Spooner & Wessells, 1970) or for the maintenance of the branching process. Previous results are not in conflict with this conclusion: Ball (1974) used 13-day\textsuperscript{1} rat lung epithelia which failed to branch in salivary mesenchyme, as did 12-day mouse lung epithelia (Taderera, 1967). 70\% of the samples of 13-day mouse lung epithelia used by Taderera underwent limited branching in salivary mesenchyme and the result was strongly influenced by the culture conditions, a situation that has been found for other heterotypic recombinates (Lawson, 1974). In the present work the results with rat heterotypic recombinates were more clear cut than those with mouse, and the stages of the rat starting material were also less variable than those of the mouse material. That the initial stage, not only of the lung epithelium but also of the salivary mesenchyme, may be important, is indicated by the age-dependent capacity of salivary mesenchyme to control epithelial curvature, (Nogawa, 1983). Differences that may lead to minor quantitative effects in control recombinates can be more important in the heterotypic recombinates.

The ability of salivary mesenchyme to maintain branching of 14-day rat lung epithelium, and then in a salivary pattern (Lawson, in preparation) is in agreement with other systems in which mesenchyme from another branching organ has been substituted for the original mesenchyme (Taderera, 1967; Kratochwil, 1969; Lawson, 1972, 1974; Ball, 1974; Sakakura \textit{et al.} 1976; Nogawa & Mizuno, 1981): the branching pattern of the epithelium resembles that expected from the mesenchyme. Epithelium in such recombinates differentiates according to its origin (Lawson, 1972; Sakakura \textit{et al.} 1976) and the recombinates of older lung epithelium with salivary mesenchyme seem to be no exception: epithelium that had branched into salivary mesenchyme resembled the cuboidal epithelium of the future respiratory portion of the lung, which is also found in homotypic recombinates; whereas the unbranched portion developed as primary bronchial epithelium. Confirmation of the equivalence of the cuboidal cells in homotypic and heterotypic recombinates awaits the identification of specific products of the respiratory epithelium, such as lamellar bodies, which have been found in homotypic lung recombinates (Masters, 1976) and an alveolar type II cell antigen which is present throughout the future respiratory epithelium \textit{in vivo} (Ten Have-Opbroek, 1979). The differentiation of the epithelium of 13-day rat primordia that had been stimulated to branch in salivary mesenchyme by removing and replacing the mesenchyme at ±65 h culture was less advanced at the time of fixation. Whether or not it develops components specific for the respiratory portion of the lung is of particular interest in the light of the recent report that

\textsuperscript{1}In this and following references to gestational stage, the convention given in Materials and Methods is followed, not that of the original author.
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salivary epithelium is able to undergo cytodifferentiation in the absence of mesenchyme only if separated from salivary mesenchyme after the onset of cleft formation (Cutler, 1980), and the implication that cytodifferentiation and the onset of branching morphogenesis are coupled.

The failure of sac-like primordia to develop in salivary mesenchyme except as primary bronchial epithelium was prevented by simply peeling off the mesenchyme after ca. 65 h culture and replacing it directly, or by renewing it with fresh salivary mesenchyme. Comparison with the results of two experiments reported by Spooner & Wessells (1970) is relevant. They found that, although lung buds derived in vitro from gut endoderm in the presence of salivary mesenchyme never (0/17) branched, lung buds derived in vitro from whole guts occasionally (5/22) did so when the original mesenchyme was scraped off and replaced with salivary mesenchyme. Two out of five positive cases showed an initial branching pattern that was more salivary- than lung-like and branching was much less extensive than in bronchial mesenchyme. Although their two experiments are not strictly comparable and the differences in incidence of response do not reach statistical significance ($\chi^2 = 2.69$, $P > 0.1$, df = 1), the parallel with the result obtained with the primordia is striking. Discussion of the effect of peeling and replacing the mesenchyme will be separated into a consideration of the characteristics of the response and the nature of the stimulus causing the response.

The epithelial response was evident as a doubling of the number of cells in S-phase within 3–5 h (the earliest time that was examined after the operation), shape changes presaging bud formation at 8–10 h, an increase in the number of mitoses during a colchicine block from 11 to 19 h, and clearly defined buds by 20–24 h, the majority of which continued to branch in a salivary fashion during further culture.

The similarity of the proportion of cells labelled with $^3$HTdR in control L13S recombinates with the non-branching part of homotypic lung recombinates, on the one hand, and that of the operated L13S recombinates with the bronchial buds of the homotypic recombinates, on the other, suggests that the labelling pattern reflects the situation in normal development. The pattern could be due to differences in cell cycle parameters in morphogenetically active and inactive regions, or to differences in the size of the population of dividing cells, or to both. Estimates of the cell cycle parameters of intact lungs in vitro did not reveal any significant differences in the phases of the cell cycle between primary bronchus and bronchial buds, but a large difference in the growth fractions: virtually all epithelial cells in the buds, but only about half in the primary bronchus, are cycling. If it is assumed that the cell cycle parameters measured in intact lungs are also valid for the recombinates, it can be concluded that differences in the proportion of labelled cells reflect differences in the populations of dividing and non-dividing cells, and that the cycling population in the buds of homotypic and responding heterotypic recombinates is about twice the size of that in the heterotypic controls and non-branching part of the homotypic recombinates.
The growth fractions of the recombinates unfortunately cannot be accurately estimated from the present data because of the labelling period of 2h, and because of the arbitrary choice of four grains/nucleus as the criterion for a labelled cell, which may have led to an underestimation of the labelling index (England & Miller, 1970).

The rapid increase in the number of S-phase cells (within 3 to 5 h at the latest) indicates a rapid mobilization of quiescent cells. The absence of any increase in colchicine-blocked mitoses during the first 11 h indicates that quiescent cells had left the cell cycle after a previous mitosis and not in G2. However, the colchicine indices should be treated with great caution as they are obviously gross under-estimates of the number of cells normally entering mitosis during 8 h. (Assuming that mitosis lasts 0.5 to 1 h, the MI of a population in which all the cells are cycling with a cell cycle time of 13 h, would be 4% to 8%, and the CI after 8 h treatment would be 61%. The highest CI found was 18% in the LL recombinates.) Considering the total length of the cell cycle (circa 13 h), cells labelled 15–17 h after operation are probably in the second cycle after stimulation. New buds that have become distinct by that time from the non-budding area of the epithelium have a high proportion of labelled cells similar to the buds of the homotypic recombinates, while the proportion in the non-budding area is at the control or non-branching level. The available material does not make it possible to distinguish whether the buds are the result of local increases in the proportion of dividing cells, present already during the first cell cycle after operation, but only recognizable after the changes in morphology are clear, or whether the whole epithelial population is initially affected and areas that are not involved in bud formation drop back to the control level after one cell cycle, while the branching portion maintains a large proportion of dividing cells as in normal development.

In combination with the area measurements the results suggest that the failure of L13S to undergo branching morphogenesis is due to a failure of salivary mesenchyme to maintain a high proportion of the epithelial cells of the primary bronchus in the cell cycle. 14-day distal buds expand more rapidly than 13-day whole primordia in salivary mesenchyme and subsequently branch, suggesting that critical changes in the regulation of epithelial growth may be occurring in vivo during the time that branching begins with the establishment of the secondary bronchial buds. This would involve the maintenance in the cell cycle of the entire epithelial population in budding regions and a gradual withdrawal in the rest of the primary bronchus, and would be reflected in the labelling indices as seen in the 14-day intact lungs in vitro, and in the homotypic, 13-day lung recombinates after 3 days culture. An analogous situation has been found in branching supernumerary buds induced by bronchial mesenchyme on the trachea (Goldin & Wessells, 1979), and there are similar differences in labelling with 3HTdR between morphogenetically active and inactive areas in avian lung (Goldin & Opperman, 1980) and mouse salivary glands (Bernfield et al. 1972).

The relative expansion of lung epithelium in its own mesenchyme (both 14-day
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During the first 3 days of culture was twice that of the same epithelium in salivary mesenchyme. No indication of a dependence of lung epithelial growth rate on the mass of associated submandibular gland mesenchyme has been found (except in so far that the mesenchyme must enclose the epithelium); this is in contrast to lung mesenchyme (Alescio & Colombo-Piper-no, 1967; Alescio & di Michele, 1968; Lawson, 1974; Masters, 1976). Under the experimental conditions used here bronchial mesenchyme therefore maintained a more rapid expansion of lung epithelium even though submandibular gland mesenchyme has been shown to promote faster growth of some other branching epithelia compared with their homotypic association (Kratochwil, 1969; Lawson, 1972). The ability of bronchial mesenchyme to support a higher growth rate in lung epithelium may explain why only bronchial mesenchyme has been found to initiate branching in supernumerary buds induced on the trachea (Wessells, 1970) and in culture-derived lung primordia (Spooner & Wessells, 1970).

Although definitive buds are not fully established until about 20 h after operation, the earliest shape changes in the epithelium that are associated with their appearance are recognizable after 8 to 10 h and have become more pronounced by 14 h. If these changes are cell cycle dependent, the timing suggests that they are occurring towards the end of S-phase, during G2 and possibly mitosis. As in other epithelia, mitotic figures are situated at the luminal surface and presumably cellular shape changes accompanying interkinetic migration during G2 and detachment of the basal surface of the cells from the basal lamina at this time (Hendrix & Zwaan, 1974) also occur in lung epithelium. Although the integrity of the epithelium would be maintained by tight junctions at the luminal surface, there must be considerable mobility of the basal parts of the cells, both with respect to their contacts with each other and with the basal lamina. If such changes are important for bud initiation, it is clear that they will only be effective if most of the epithelial cells in the area involved are cycling and/or are not randomly distributed through the cell cycle. A closer analysis of cell cycle parameters, epithelial cell behaviour and the dynamic aspects of the basal lamina in L13S recombinates would be necessary to test this.

The nature of the stimulus provided by peeling and reassociating L13S recombinates is obscure: what the stimulus is not, is clearer than what it is. That the increase in the proportion of epithelial cells in S-phase is not the result of wounding or unspecific spatial reorganization is shown by the drop in the CI of epithelia without restored mesenchyme, the unchanged CI of operated mesenchyme, and the absence of significant shape changes or a rise in the number of cells labelled with 3HTdR in peeled epithelia recombined with stomach mesenchyme. It is also clear that no irreversible changes have occurred in salivary mesenchyme during ca. 65 h culture in association with 13-day lung epithelium.

There are various possibilities. Firstly, extracellular material such as collagen, proteoglycan or a stable basal lamina in the epithelial-mesenchymal interface
could prevent the passage of growth factors from salivary mesenchyme, or the
close association of mesenchyme with epithelium that may be necessary for a
high growth rate. Removal or damage of the interface material by peeling
would allow the growth stimulus to pass. Alternatively, the operation could
stimulate the mesenchyme to accelerate the turnover of interface material that
also occurs in normal salivary development (for recent review see Bernfield,
1981; also Bernfield & Banerjee, 1982) and thus produce the conditions
necessary for bringing quiescent epithelial cells back into the cell cycle. Al-
though this interpretation conforms to current views on growth control in
branching organs (Goldin, 1980), no striking alterations in the interface have
been found before, or concomitant with, the rise in the labelling index
(Lawson, in preparation).

Secondly, the operation may stimulate salivary mesenchyme to produce
epithelial growth stimulators, or may remove a mesenchyme-associated in-
hibitor.

Thirdly, the spatial organization of salivary mesenchyme may determine its
growth-promoting capacity. The epithelium in 65 h-old L13S cultures is surro-
ded by elongated, rather closely packed mesenchyme cells (Fig. 10C), while
those further out are more diffuse and similar to the mesenchyme surrounding
salivary buds. If the growth-promoting capacity is associated with the diffuse
cells, disruption of the mesenchymal sheath round the epithelium and reassoci-
ation of the epithelium with other areas of mesenchyme may be sufficient to
reactivate quiescent cells.

Fourthly, alterations in epithelial cell shape caused by the release of the
epithelial cyst from the restraining mesenchyme and the slight flattening of the
cyst on the substratum at reassociation could expose quiescent cells to growth
stimulation. The main objection to this is that, although the same epithelial
alterations would occur in the operated controls with stomach mesenchyme, this
mesenchyme maintains the dividing population at the same level as that in the
unoperated controls but does not stimulate quiescent cells. However, if the early
shape changes are not cell cycle dependent, they could be the result of the
reassociated salivary mesenchyme increasing the curvature of the flattened
epithelial cyst, as in the mechanism proposed by Nogawa (1982) for normal
salivary development.

In conclusion, it is suggested that the stage dependency in mesenchyme
requirement shown by lung primordia is connected with the ability of the
associated mesenchyme to maintain most or all of the epithelial cells in the cell
cycle, and that a high proportion of dividing cells in an epithelial population is
a prerequisite for the further interaction of epithelium and mesenchyme leading
to branching morphogenesis.

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