J. Embryol. exp. Morph. Vol. 32, 2, pp. 469–493, 1974
Printed in Great Britain

Mesenchyme specificity in rodent salivary gland development: the response of salivary epithelium to lung mesenchyme *in vitro*

By KIRSTIE A. LAWSON[1]
From the Hubrecht Laboratory, The Netherlands

SUMMARY

Lung mesenchyme is able to support budding and cytodifferentiation of salivary epithelial rudiments *in vitro*. No difference in response was found between submandibular and parotid epithelium from mouse or rat.

There are several further features of this result, which is contradictory to previous findings. (1) Lung mesenchyme is quantitatively less effective than submandibular mesenchyme for supporting submandibular morphogenesis. At least part of this difference is attributed to the inability of submandibular epithelium to replace lung epithelium in supporting the growth of lung mesenchyme. (2) Rat lung mesenchyme is quantitatively more effective than mouse lung mesenchyme when recombined with mouse submandibular epithelium. This may be at least partly due to mouse lung being more easily damaged by the procedures used. (3) Whereas the response of submandibular epithelium to submandibular mesenchyme is equally good on an agar or Millipore filter (MF) substratum, the response to lung mesenchyme is severely reduced or eliminated on MF. This difference is interpreted in terms of different mesenchymal cell densities necessary for submandibular or lung mesenchyme to support branching morphogenesis.

Salivary buds formed in lung mesenchyme after 6 days are smaller and more closely packed than in salivary mesenchyme. In these heterotypic recombinates, the accumulation of amylase-resistant, PAS-positive material in the buds is initially accelerated and the tubular epithelium accumulates glycogen.

INTRODUCTION

The ability of mouse submandibular gland epithelium to undergo branching morphogenesis *in vitro* has been so far held to depend on a specific requirement for salivary mesenchyme: morphogenesis has been found to occur only in the presence of mouse submandibular (Grobstein, 1953) and parotid (Grobstein, 1967), or chick submandibular (Sherman, 1960) mesenchyme, but not in lung (Grobstein, 1953; Spooner & Wessells, 1972), mammary (Kratochwil, 1969), metanephric, mandibular arch, or limb-bud mesenchyme (Grobstein, 1953). In contrast, mouse submandibular mesenchyme is able to support the development of non-salivary epithelia, such as that of the thymus (Auerbach, 1960),

[1] *Author's address:* Hubrecht Laboratory, p/a Universiteits Centrum 'de Uithof', Uppsalalaan 1, Utrecht, The Netherlands.

pancreas (Golosow & Grobstein, 1962; Fell & Grobstein, 1968) and mammary gland (Kratochwil, 1969). Such results led to the proposal that salivary mesenchyme possesses both a mesenchyme common factor or property, shared with other mesenchymes, and a mesenchyme specific factor or property unique to salivary mesenchyme and essential for salivary morphogenesis (Grobstein, 1967).

In apparent contrast to mouse submandibular, rat parotid epithelium was shown to be able to undergo morphogenesis and functional differentiation in rat lung mesenchyme (Lawson, 1972). Also, Cunha (1972) has shown that mouse submandibular epithelium develops extensively in mesenchyme from male secondary sex organs when the recombinates are cultured in the anterior chamber of the eye.

These results made desirable a reinvestigation of mesenchyme requirement in salivary development. Attention has been concentrated on recombinates of salivary epithelium with lung mesenchyme *in vitro*: the relative importance of differences within the salivary system, species differences, mesenchyme mass, and culture conditions has been assessed.

MATERIALS AND METHODS

Animals

Wistar rats and Swiss mice were used. In calculating the embryonic age, the morning on which vaginal sperm (rats) or a copulation plug (mice) were found after overnight mating was counted as day 1.

Tissues

Parotid glands from 17-day-old foetuses of rats and 15-day-old foetuses of mice, submandibular glands from 16-day-old foetuses of rats and 14-day-old foetuses of mice, and lungs from 13- and 14-day-old foetuses of rats and 12- and 13-day-old foetuses of mice were used. The morphogenetic stages of these organs are shown in Fig. 1.

Tissue culture

As described previously (Lawson, 1972), the rudiments were separated into epithelial and mesenchymal components by trypsin–pancreatin treatment and dissection, recombined on an agar film which was supported by a frame of cellulose acetate net, and cultured on a medium of cock plasma and chick embryo extract in a humidified atmosphere of air and 5 % CO_2. Cultures that were maintained for 12 days were transferred to 45 % N_2, 50 % O_2 and 5 % CO_2 on the 9th day. Unless mentioned otherwise the epithelium from one salivary rudiment was recombined with mesenchyme from two salivary glands or lungs. In experiments in which recombinates were made directly on the platform of a Millipore filter (MF) assembly (modified from Grobstein, 1956) the nutrient

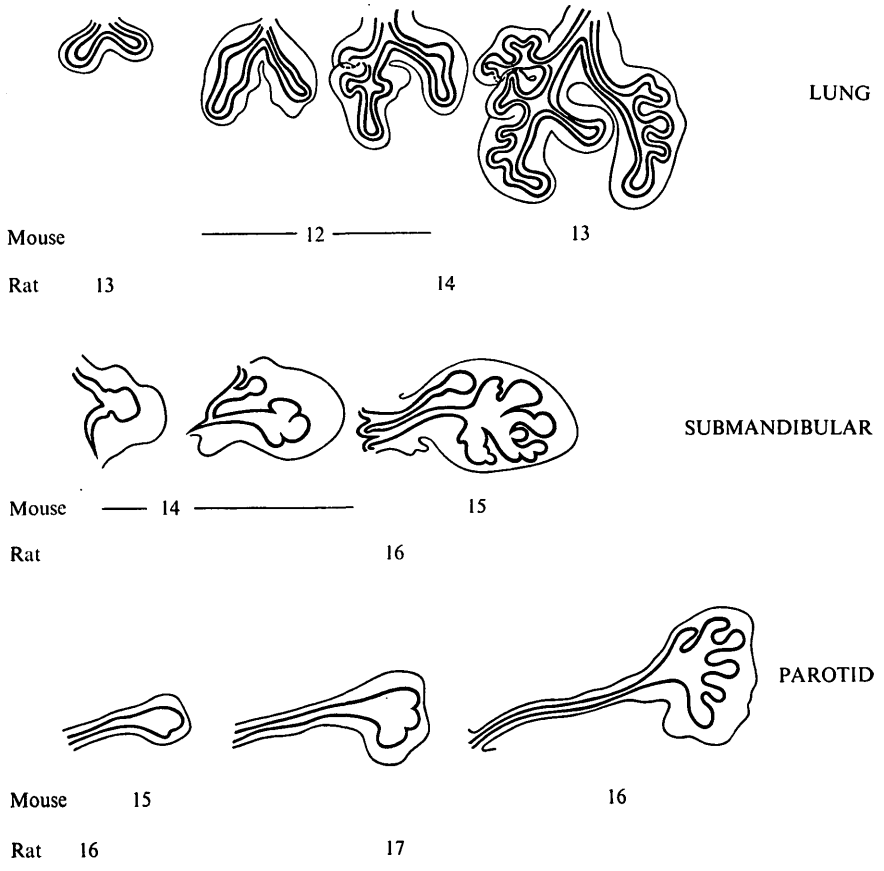

Fig. 1. Morphogenetic stages of salivary glands and lungs used for recombination. Numbers refer to days of gestation.

medium (referred to as liquid medium) was Ham's F 12 with 100 i.u. penicillin and streptomycin/ml, 10 % foetal bovine serum, and 10 % chick embryo extract.

Histology

Tissues were fixed in Helly's or Carnoy's fluid and sections were stained with PAS (periodic acid–Schiff), alcian blue at pH 2·8, and Mayer's haemalum. Glycogen was detected by comparing amylase-treated sections with adjacent control sections. The sections were incubated for 30 min at 37 °C in 3 i.u. amylase (Worthington, 3 × crystallized)/ml 0·02 M phosphate buffer with 0·007 M sodium chloride at pH 6·8.

In one series of experiments the critical electrolyte concentration–alcian blue method for acidic glycosaminoglycans (Scott & Dorling, 1965) was used.

Criteria for salivary development

(1) *Morphogenesis.* A quantitative estimate of epithelial morphogenesis was obtained by counting the number of buds in camera lucida tracings of the

explants drawn under standardized conditions of lighting and magnification. Buds were defined on the basis of indentation of the epithelial outline into the epithelium, rather than extension of the epithelium into the mesenchyme: the two sides of the indentation were required to have an angle of less than 135° on the mesenchymal side; each side would then belong to a different bud. This method underestimates newly initiated buds that have no lateral extension in the plane of the drawing; well-formed vertical clefts are easily visible and were assumed to have an angle of less than 135°. The method is unreliable after 5–6 days of culture when fast-growing recombinates have thickened and buds overlap.

In one experiment the volumes of epithelium and mesenchyme in the recombinates after 6 days culture were measured from sections using a point grid method. The number of mesenchyme cells per unit volume was measured by counting mesenchyme nuclei in 12 standard areas of mesenchyme (2328 μm^2) per sectioned explant.

(2) *Cytodifferentiation.* The presence of amylase-resistant, PAS-positive material was scored since such material accumulates in the terminal buds towards the end of the foetal period in the submandibular (Gerstner, Flon & Butcher, 1963; Szymanska, 1963; Di Giovine Vecchione, 1967) and perinatally in the parotid (Bignardi, 1961; Shubnikova & Chunaeva, 1966; Lawson, 1970; Redman & Sreebny, 1971) and also in both glands *in vitro* (Di Giovine Vecchione, 1967; Lawson, 1970, 1972). No such accumulation has been found in the epithelium *in vitro* in the absence of morphogenesis.

Mesenchymal protein

The initial protein content of representative samples of mesenchyme was determined by the Folin–Lowry procedure (Lowry, Rosebrough, Farr & Randall, 1951).

Statistical analysis

The distribution of attributes of morphogenesis and of cytodifferentiation were tested with the χ^2 test.

The effects of different treatments on the number of epithelial buds present at any one time, on the final volumes of epithelium and mesenchyme, and on the number of mesenchyme cells per explant were tested by analysis of variance after transformation of the data to the logarithmic scale. This transformation was justified since the variances of the means after different treatments differed significantly before, but not after, transformation. The transformation was not required for analysing the data on cells per unit volume, which appeared to be normally distributed with similar variances. A randomized block design was used for all experiments in which analysis of variance was applied.

Table 1. *Morphogenesis and cytodifferentiation of recombinates*

	Epithelium	Mesenchyme	Morphogenesis after 5 days +	Morphogenesis after 5 days −	Amylase-resistant PAS in buds, 9–12 days +	Amylase-resistant PAS in buds, 9–12 days −
Rat	Parotid	Parotid	9	2	8	3
	Parotid	Lung	10	1	10	1
	Submandibular	Submandibular	10	2	8	2
	Submandibular	Lung	9	1	8	2
Mouse	Parotid	Parotid	17	2	19	0
	Parotid	Lung	19	3	13	3
	Submandibular	Submandibular	27	0	26	0
	Submandibular	Lung	28	2	24	5
Mouse epithelium + rat mesenchyme	Parotid	Lung	6	0	6	0
	Submandibular	Lung	7	0	7	0

RESULTS

Development of salivary epithelium in lung mesenchyme

Parotid and submandibular epithelia from both mouse and rat were recombined with their own mesenchyme and with homospecific lung mesenchyme (14-day-old foetuses of rat, 12- and 13-day-old foetuses of mouse).

Morphogenesis occurred in all types of recombinate (Table 1) and there was no significant difference in the proportion showing morphogenesis between homotypic and heterotypic recombinates in either species, or between parotid and submandibular epithelium. However, mouse salivary/lung recombinates did not increase in overall size after 2–3 days, but began to lose cells and finally became very small. After continued culture, cytodifferentiation was found in all types of recombinate. Rat salivary epithelium differentiated as well in lung as in its own mesenchyme (Table 1; Fig. 2A, B); mouse salivary epithelium differentiated in mouse lung mesenchyme (Table 1; Fig. 2C, D), but there was a significant number of negative results ($\chi^2_c = 6{\cdot}72$, degrees of freedom = 1, $P < 0{\cdot}01$), presumably due to the progressive loss of mesenchyme cells.

Heterotypic, heterospecific recombinates of mouse salivary epithelium with rat lung mesenchyme were also made. These did not lose cells from the edge of the explant or diminish in size after three days: their morphogenesis and cytodifferentiation were comparable to the heterotypic, homospecific rat recombinates (Table 1; Fig. 2E). This suggests a difference in behaviour between rat and mouse lung mesenchyme, rather than a difference between rat and mouse salivary epithelia in their ability to respond to lung mesenchyme.

Since both parotid and submandibular epithelium from both rat and mouse

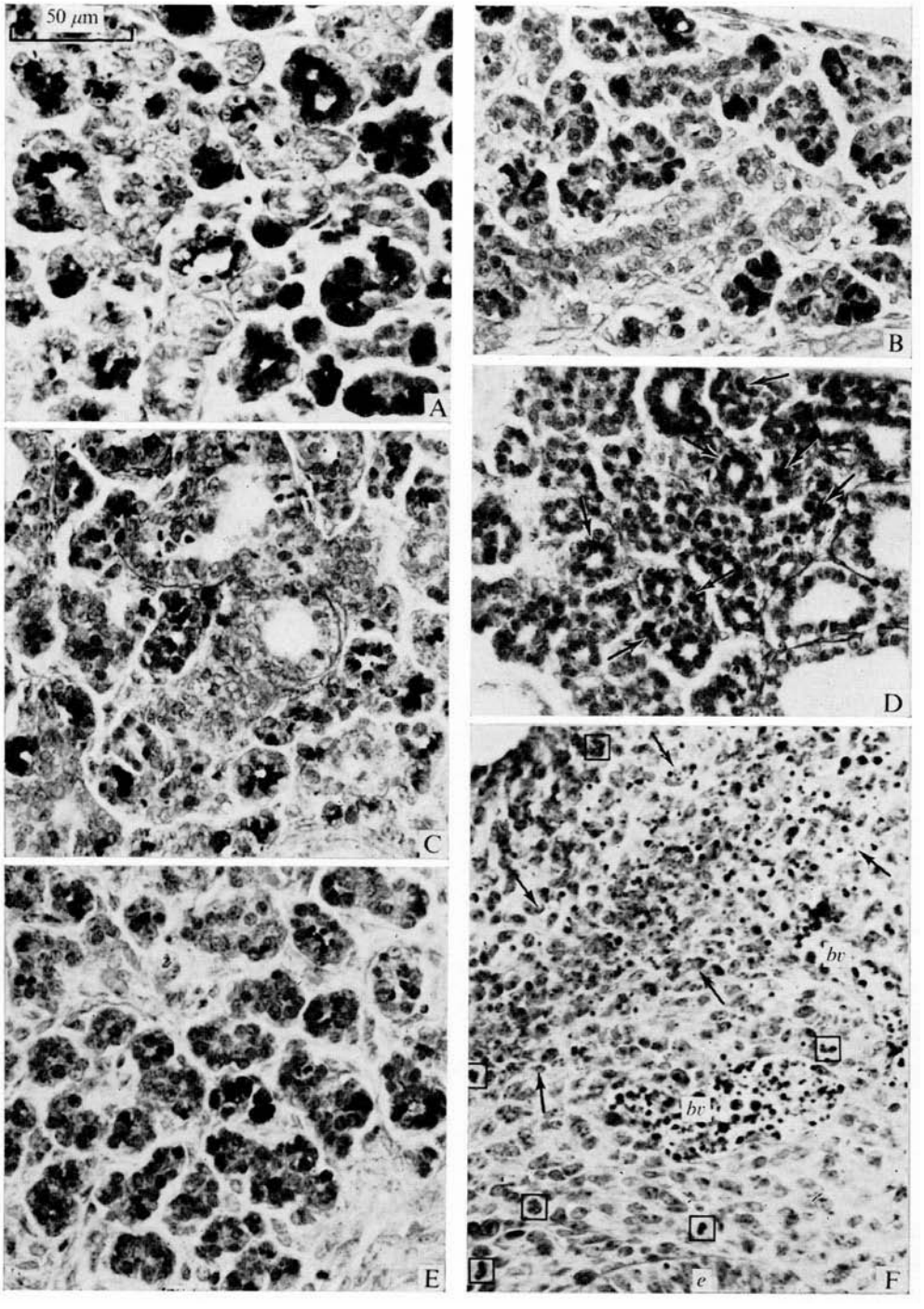
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Table 2. *Initial protein content of mesenchyme*

Source	Tissue	μg protein/-mesenchyme	S.D.	*n*
Mouse	14-day submandibular	2·86	0·57	11
	12-day lung	2·80*	1·06	8
	13-day lung	6·78*	1·82	8
Rat	13-day lung	1·56*	0·25	9
	14-day lung	4·81*	0·63	9

S.D. = standard deviation. *n* = number of samples.
* Value for half of a pair of lungs.

are able to develop in lung mesenchyme it is concluded that there is no mesenchyme-specific factor unique to salivary mesenchyme and essential for salivary epithelial morphogenesis.

Effect of mesenchyme mass on submandibular morphogenesis

(*a*) *Mouse recombinates*

The possibility that the initial mass of mesenchyme in the recombinate is a critical factor for supporting morphogenesis was tested by combining the epithelium from one mouse submandibular rudiment with the mesenchyme from one, two, three or four submandibulars, 12-day lungs, or 13-day lungs. 'One' lung mesenchyme was defined as half the mesenchyme obtained from a pair of lungs (the right lung is about twice the size of the left lung at the stages used). The protein content of mesenchyme from all three sources was measured in other prospective litters (Table 2), showing that the mesenchyme from 'one'

FIGURE 2

A–E. Accumulation of amylase-resistant, PAS-positive material in recombinates after 12 days culture. Helly fixation. Scale line refers to A–F.

(A) Homotypic recombinate of rat submandibular. Dark areas in epithelial cytoplasm are PAS-positive.

(B) Heterotypic recombinate of rat submandibular epithelium with lung mesenchyme. Abundant PAS-positive material in terminal buds.

(C) Homotypic recombinate of mouse submandibular. Abundant PAS-positive material.

(D) Heterotypic recombinate of mouse submandibular epithelium with lung mesenchyme. Arrows indicate PAS-positive material.

(E) Heterotypic, heterospecific recombinate of mouse submandibular epithelium with rat lung mesenchyme. Abundant PAS-positive material.

(F) Heterotypic recombinate of mouse submandibular epithelium with mouse lung mesenchyme after 16 h culture. Mesenchyme adjacent to the epithelium (*e*) is healthy, a strip (arrows) peripheral to the blood vessels (*bv*) contains many necrotic cells. Squares enclose mitotic cells. Carnoy fixation.

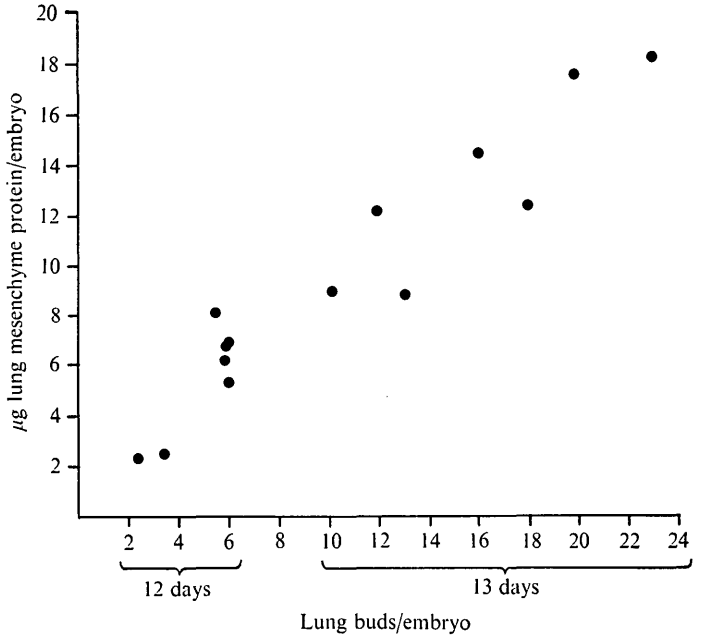


Fig. 3. Correlation of protein content of mouse lung mesenchyme with the number of epithelial buds present in the intact lungs.

13-day lung had approximately twice the amount of protein as 'one' 12-day lung or 14-day submandibular. The high variation in the values for lung mesenchyme appears to be due to the very rapid growth of this organ, since the average protein content of the mesenchyme is correlated with the average number of epithelial buds per pair of lungs (Fig. 3).

The experiment was set up as a 4×3 factorial in three replicates, with duplicates within each replicate; N (total number of explants) = 72.

After the initial rounding-up, the epithelium expanded considerably in lung mesenchyme forming large, flat buds which were then further subdivided by clefts (Fig. 4C, D). In inadequate amounts of mesenchyme this expansion was restricted and subsequent morphogenesis was negligible (Fig. 4B). The diminishing overall size of the heterotypic recombinates after the second day of culture, compared with the continued growth of the homotypic recombinates, is also evident in Fig. 4. A cyst appeared in the epithelium between the second and third day and by the sixth day had expanded, excluding the buds to the periphery.

Varying the initial mass of submandibular mesenchyme had no effect on the rate of bud formation (Fig. 5A). However 'two', 'three' and 'four' 13-day lung mesenchymes supported substantial bud formation, 'one' significantly less (Fig. 5C). 'Three' and 'four' 12-day lung mesenchymes supported substantial epithelial morphogenesis, 'two' significantly less, whereas 'one' 12-day lung mesenchyme failed to support significant morphogenesis (Fig. 5B), although

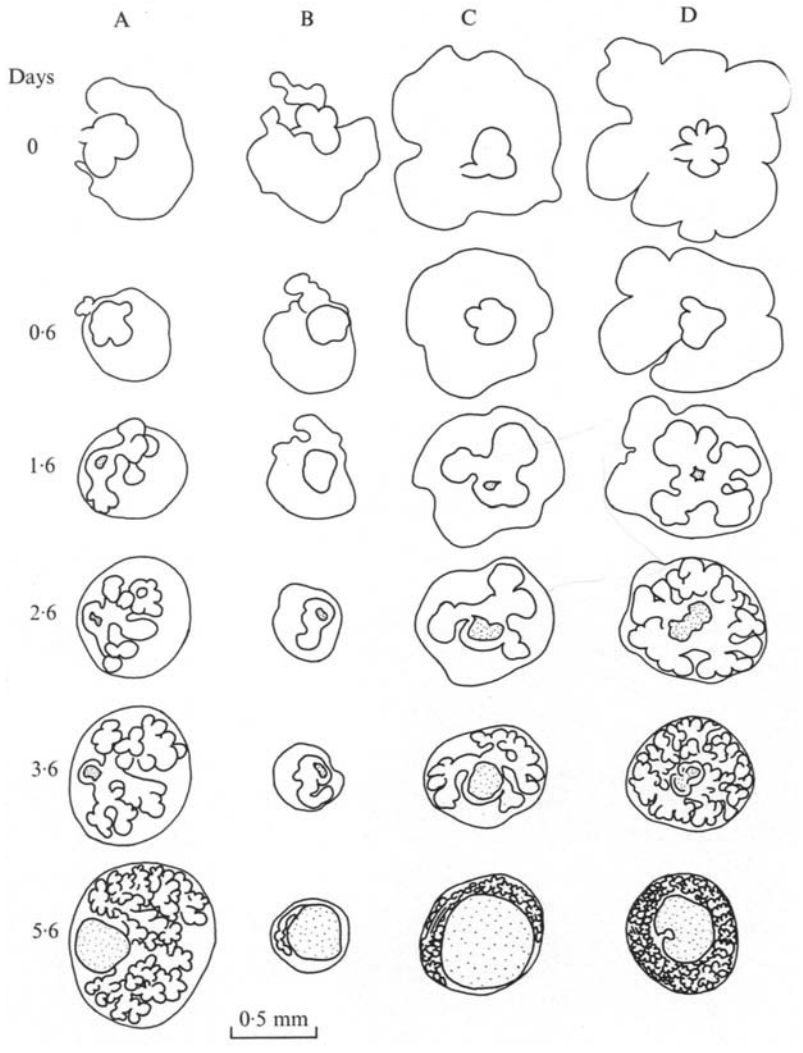


Fig. 4. Camera lucida tracings, from the same replicate, of living recombinates of mouse submandibular epithelium with mesenchyme from (A) one submandibular, (B) 'one' 12-day lung, (C) 'three' 12-day lungs, (D) 'two' 13-day lungs. Lumen in epithelium stippled.

the initial amount of mesenchyme present, estimated as protein, was about the same as one submandibular mesenchyme.

Thus relatively more mouse lung mesenchyme than submandibular mesenchyme is needed to support morphogenesis of submandibular epithelium.

(*b*) *Mouse recombinates on MF and liquid medium*

If the previously reported failure of mouse lung mesenchyme to support submandibular epithelial morphogenesis (Grobstein, 1953; Spooner & Wessells,

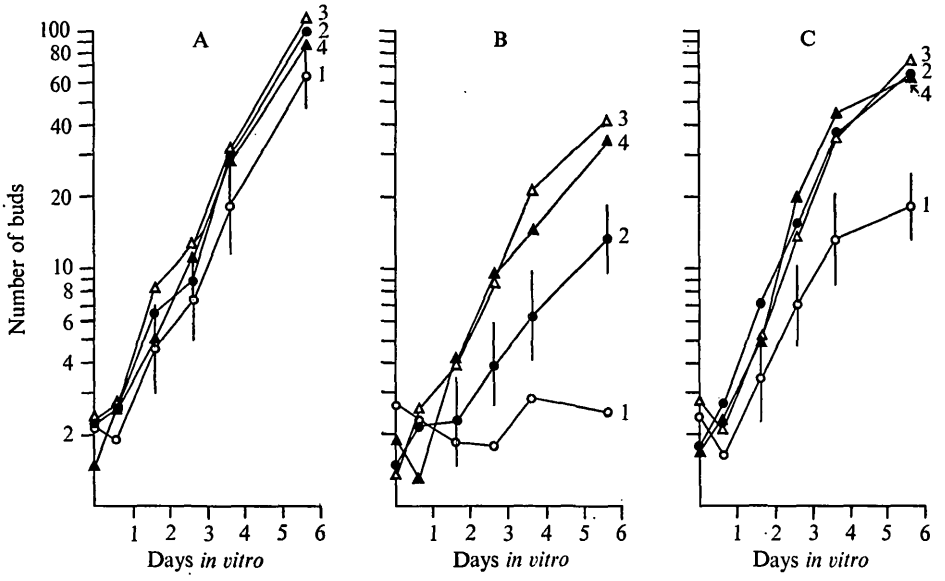


Fig. 5. Effect of mesenchyme mass in homo- and heterotypic recombinates on the morphogenesis of mouse submandibular epithelium. Numbers indicate relative initial mesenchyme masses for each type of recombinate. (A) Homotypic recombinate with submandibular mesenchyme. (B) Heterotypic recombinate with 12-day lung mesenchyme. (C) Heterotypic recombinate with 13-day lung mesenchyme. Vertical lines indicate the 95 % confidence limits for the means at any one time.

1972) was due solely to insufficient mesenchyme, the presence of large quantities of lung mesenchyme should ensure morphogenesis under the same culture conditions. Culture conditions similar to those used by Spooner & Wessells (MF over liquid medium) were chosen since these are currently in general use for organ cultures. Mouse submandibular epithelium was recombined with 'two', 'four' or 'six' 12-day lung mesenchymes. Homotypic control recombinates of submandibular epithelium with two submandibular mesenchymes and of left lung epithelium with 'two' lung mesenchymes were also made.

Increasing initial amounts of lung mesenchyme supported some morphogenesis of submandibular epithelium (Fig. 6), but much less than was expected. When epithelial morphology was classified into three groups (−, no buds (Fig. 6A); ±, buds but no branching (Fig. 6B); +, branched buds (Fig. 6C)) it was found that morphogenesis was better in 'four' and 'six' lung mesenchymes than in 'two' (Table 3; χ^2 for heterotypic recombinates, 2 vs. 4 = 14·92, 2 vs. 6 = 14·24, $P < 0{\cdot}001$, degrees of freedom = 2).

Thus a relatively large quantity of mouse lung mesenchyme is necessary for submandibular epithelium to achieve a very limited morphogenesis when the recombinates are supported by MF over liquid medium.

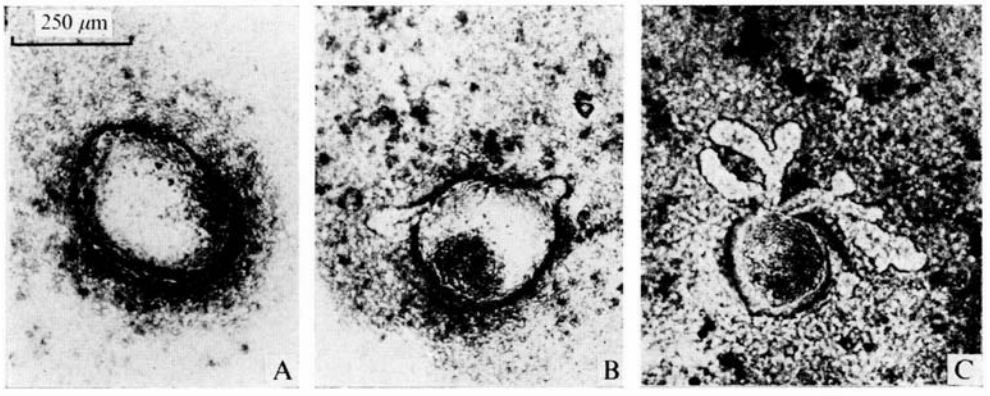


Fig. 6. Mouse submandibular epithelium recombined with varying amounts of mouse lung mesenchyme and cultured on MF and liquid medium for 6 days. (A) In mesenchyme from 'two' lungs, (B) in mesenchyme from 'four' lungs, (C) in mesenchyme from 'six' lungs.

Table 3. *Morphogenesis of mouse submandibular epithelium combined with varying amounts of lung mesenchyme and cultured on MF over liquid medium for 6 days*

Epithelium ...		Submandibular				Submandibular				Lung			
Mesenchyme ...		Lung				Submandibular				Lung			
Morphogenesis*		−	±	+	Total	−	±	+	Total	−	±	+	Total
Initial†	2	10	2	0	12	0	0	8	8	2	0	5	7
mesenchyme	4	0	6	3	9								
mass	6	2	0	7	9								

* Nos. of explants showing: −, no buds; ±, primary buds; +, branched buds.
† See p. 475.

(*c*) *Mouse submandibular epithelium in rat lung mesenchyme*

Unlike the recombinates of mouse salivary epithelium in mouse lung mesenchyme, which became smaller after 2 or 3 days on agar and plasma-embryo extract, recombinates of rat or mouse salivary epithelia in rat lung mesenchyme maintained their size and did not lose mesenchyme cells (p. 473), suggesting that mouse and rat lung mesenchymes differ in their interaction with salivary epithelium.

To test whether 14-day rat lung mesenchyme is quantitatively equivalent to mouse submandibular mesenchyme for mouse submandibular epithelial development, a half, one and two masses of each were recombined with mouse submandibular epithelium in a 3×2 factorial experiment in three replicates, with triplicates within each replicate; $N = 54$. In addition, a half, one and two masses of lung mesenchyme were recombined with the epithelium of the left lung. Equivalent masses of mesenchyme were taken on the basis of the

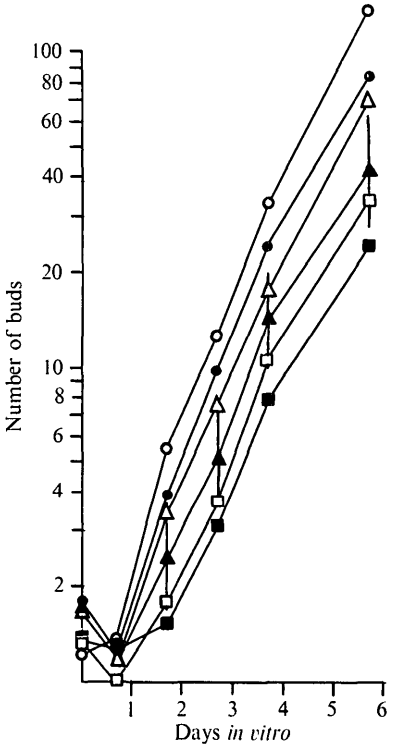


Fig. 7. Morphogenesis of mouse submandibular epithelium in equivalent initial masses of its own (open symbols) and rat lung (solid symbols) mesenchyme. Unit mass relates to the protein content of one submandibular mesenchyme: squares, one half mass; triangles, one mass; circles, two masses. Vertical lines indicate the 95% confidence limits for the means at any one time.

expected protein content (Table 2) and subsequently checked on random samples from the same litters as used in the experiment. The estimated initial protein content per unit mass of mesenchyme (based on that from one submandibular) for the three replicates was: submandibular – 2·90 μg, 3·99 μg, 2·65 μg, mean 3·18 μg; lung – 2·46 μg, 3·23 μg, 3·05 μg, mean 2·91 μg. The effect of initial mesenchymal mass on bud number (Fig. 7; $P < 0{\cdot}001$ at 2, 3, 4 and 6 days) and on final epithelial volume (Fig. 8; $P < 0{\cdot}001$) may be misleading, since the smaller masses of mesenchyme were not able to enclose the epithelium completely. The number of salivary buds was 37% higher in submandibular than in lung mesenchyme at 2 days ($P < 0{\cdot}05$), 30% higher at 3 and 4 days ($P < 0{\cdot}1$) and 55% higher at 6 days ($P = 0{\cdot}01$).

The final difference between homo- and heterotypic recombinates was even more pronounced when the volumes of epithelium after 6 days culture were compared (Fig. 8): the volume of salivary epithelium present in lung mesenchyme was only 30–50% of that in submandibular mesenchyme ($P < 0{\cdot}001$). The final volume of lung mesenchyme in the heterotypic recombinates also was

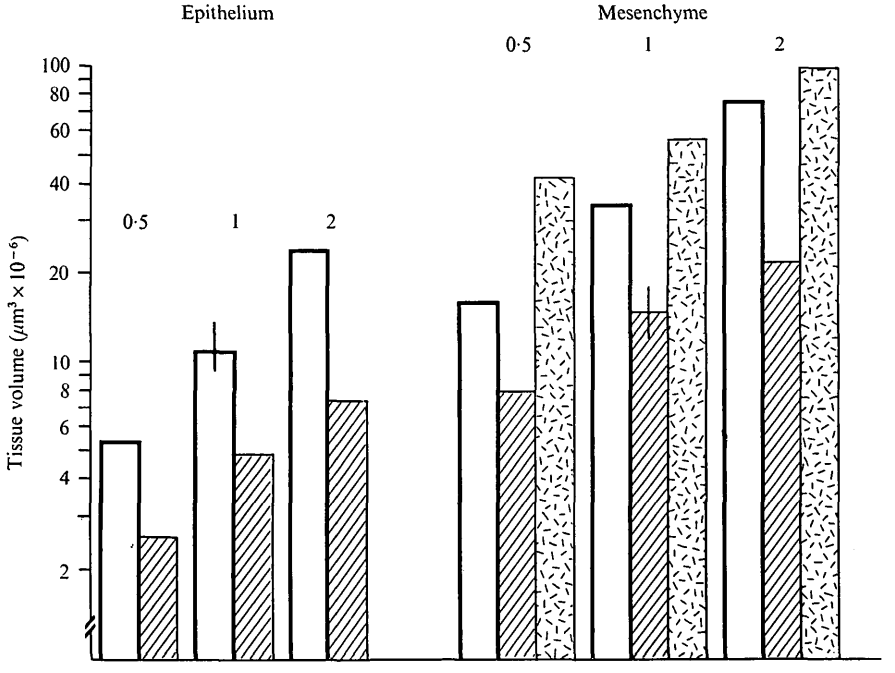
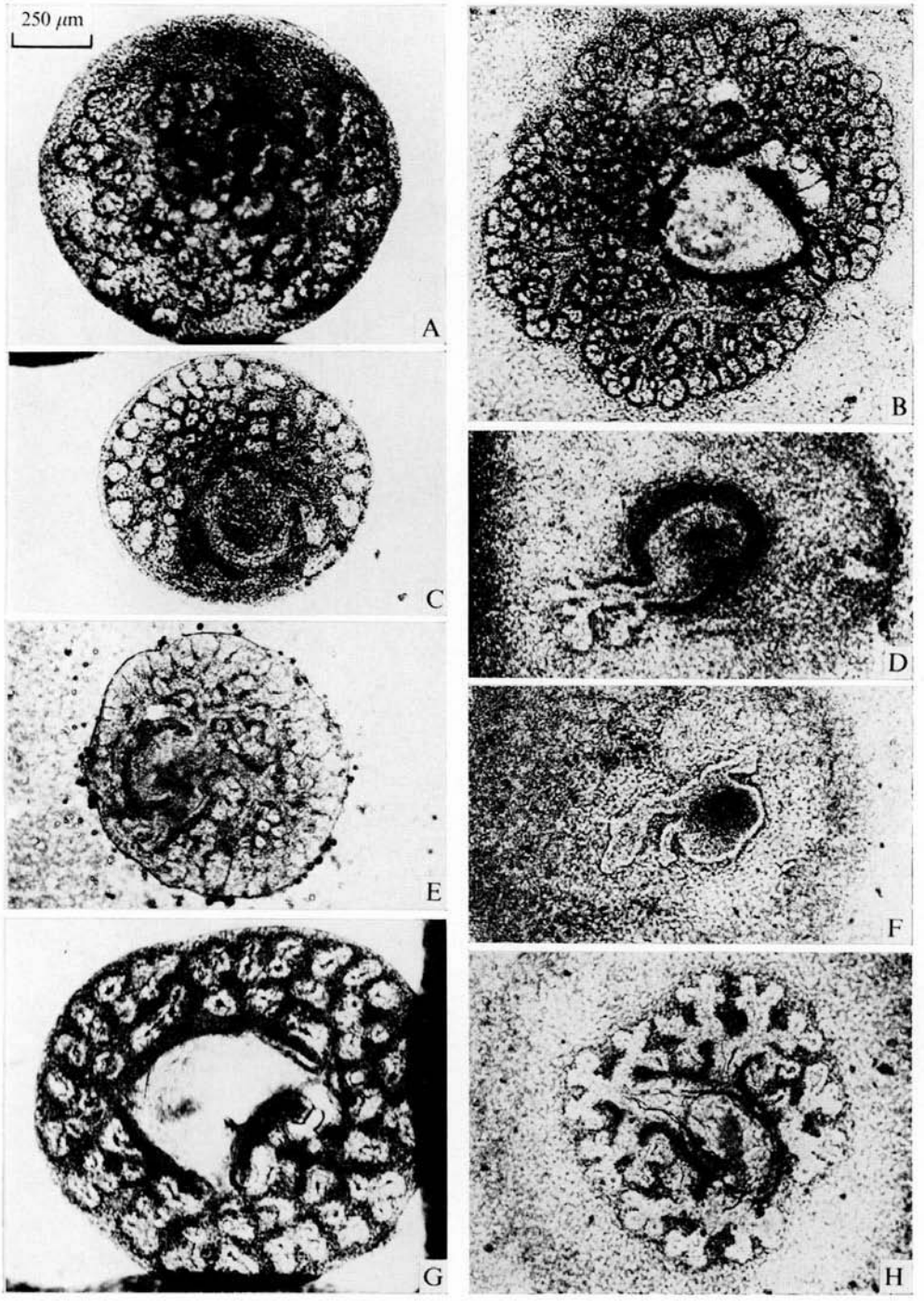


Fig. 8. Volumes of epithelium and mesenchyme after 6 days culture as recombinates of mouse submandibular epithelium with its own mesenchyme (open columns) or with rat lung mesenchyme (hatched columns), and of rat lung epithelium with its own mesenchyme (stippled columns – only mesenchyme shown). Vertical lines indicate the 95 % confidence limits.

Table 4. *Mesenchyme cells in recombinates with two mesenchyme masses (mouse submandibular or rat lung) after 6 days culture, fixed in Helly's fluid (n = 6)*

Recombinate		Mesenchyme			
		Cells/10^5 μm^3		Cells × 10^{-3}/explant	
Epithelium	Mesenchyme	Mean	95 % confidence limits	Mean	95 % confidence limits
Submandibular	Submandibular	130	99–161	104	83–124
Submandibular	Lung	225	194–256	51	43–60
Lung	Lung	226	195–257	218	183–259

only 30–50 % of the submandibular mesenchyme in the homotypic recombinates ($P < 0{\cdot}001$) and 19–26 % of mesenchyme in the homotypic lung recombinates ($P < 0{\cdot}001$). Taking into account the data showing that lung mesenchyme cells are more densely packed, in both homo- and heterotypic recombinates, than submandibular mesenchymal cells (Table 4), calculation showed that the heterotypic recombinates contained half as many mesenchyme cells as the homotypic submandibular recombinates, which again had half as many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mesenchyme cells as the homotypic lung recombinates (Table 4, $P < 0{\cdot}001$ for all comparisons).

It is concluded that small amounts of rat lung mesenchyme (equivalent in mass to half a mouse submandibular mesenchyme) can support submandibular budding. However, the reinitiation of budding by submandibular epithelium after trypsinization is slower in rat lung mesenchyme than in homospecific, submandibular mesenchyme. An interaction between epithelium and mesenchyme is involved in the growth of both components in heterotypic recombination.

Effect of substratum and medium

The very limited morphogenesis obtained in heterotypic mouse recombinates cultured on MF over liquid medium suggests that either (or both) substratum or medium interfered with the epithelial–mesenchymal interaction. These possibilities were tested using mouse submandibular epithelium in combination with rat lung mesenchyme since rat mesenchyme is quantitatively more effective than that of mouse on the agar/plasma clot medium. Mesenchyme with an estimated initial protein content equivalent to two submandibular mesenchymes was used throughout. The four combinations agar/plasma clot, agar/liquid medium, MF/plasma clot, and MF/liquid medium, were tested in a (2×2) factorial experiment ($N = 20$). For the second combination a drop of agar was allowed to gel on the platform of a MF assembly; for the third combination a small piece of MF was placed on the surface of the plasma clot. Homotypic recombinates of submandibular and lung were tested with the first and last combination only.

The results were unequivocal (Figs. 9, 10): submandibular epithelium showed

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FIGURE 9

Effect of substratum and medium on homotypic and heterotypic recombinates after 5 days culture. Scale line refers to A–H.

(A, B) Mouse submandibular epithelium in its own mesenchyme.

(A) On agar over plasma-embryo extract clot.

(B) On Millipore filter over liquid medium.

(C–F) Mouse submandibular epithelium in rat lung mesenchyme.

(C) On agar over plasma-embryo extract clot.

(D) On Millipore filter over plasma-embryo extract clot.

(E) On agar over liquid medium.

(F) On Millipore filter over liquid medium.

(G, H) Rat lung epithelium in its own mesenchyme.

(G) On agar over plasma-embryo extract clot.

(H) On Millipore filter over liquid medium.

Dark patches at the edges of A, C and G are due to the cellulose acetate net support of the agar film.

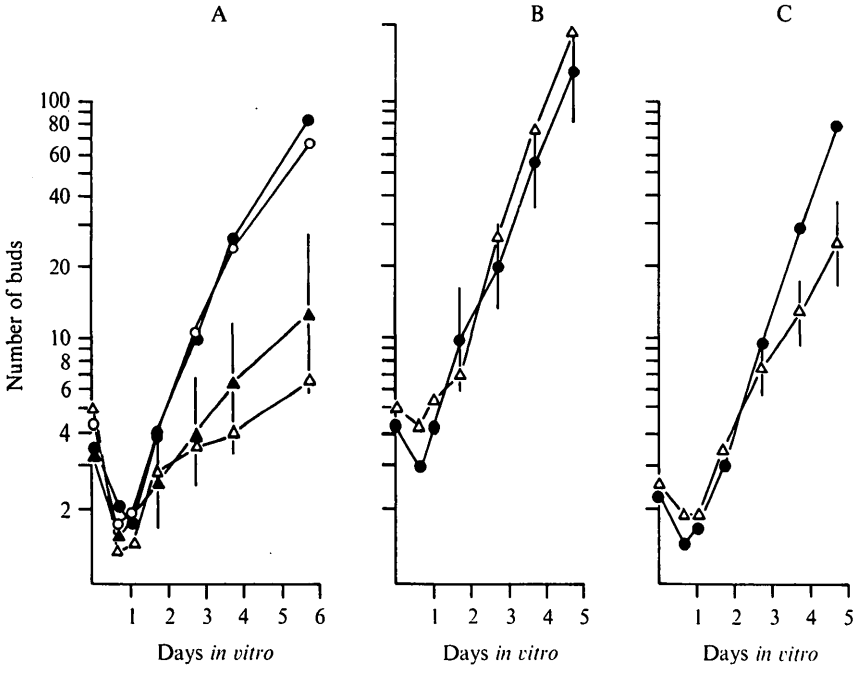


Fig. 10. Effect of substratum and medium on epithelial budding of homotypic and heterotypic recombinates. (A) Mouse submandibular epithelium in rat lung mesenchyme, (B) mouse submandibular epithelium in its own mesenchyme, (C) rat lung epithelium in its own mesenchyme. Circles, agar substrate; triangles, Millipore filter; solid symbols, plasma-embryo extract clot; open symbols, liquid medium. Vertical lines indicate 95 % confidence limits.

good morphogenesis in lung mesenchyme only when the recombinate was supported by agar, irrespective of the nutrient medium (Figs. 9C, E, 10A) whereas only meagre budding occurred on MF (Fig. 9D, F). No such differences between agar and MF were found for the homotypic submandibular recombinates (Figs. 9A, B, 10B) and there was only a limited, though definite effect ($P < 0{\cdot}001$ at 4 and 5 days) on the homotypic lung recombinates (Figs. 9G, H, 10C).

Comparison of cell density in the mesenchyme after 6 days culture (Table 5) showed no significant difference between cell spacing in submandibular mesenchyme on the two substrata. However, the cells in lung mesenchyme were closely packed when on agar but became diffuse on MF, more so in the heterotypic than in the homotypic recombinates ($P < 0{\cdot}001$).

Effect of initial age of lung mesenchyme

Fourteen-day rat lung is generally more developed than 12-day mouse lung. The possibility that a critical stage exists before which lung mesenchyme is unable to support mouse submandibular morphogenesis was tested in recombinates with 13-day rat lung mesenchyme. Four mesenchymes per

Table 5. *Mesenchymal cell density in recombinates (mouse submandibular, rat lung) cultured for 6 days on agar and plasma-embryo extract or on MF and liquid medium; fixed in Helly's fluid*

Recombinate		Mesenchyme cells/10^5 μm^3	
Epithelium	Mesenchyme	Agar	MF
Submandibular	Submandibular	86	84
Submandibular	Lung	238	123
Lung	Lung	228	159

S.E. from analysis of variance, of difference between any two means = 8·7, degrees of freedom = 17.

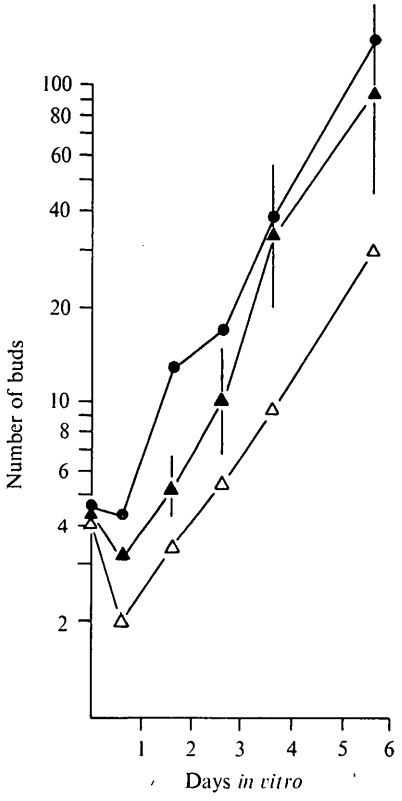
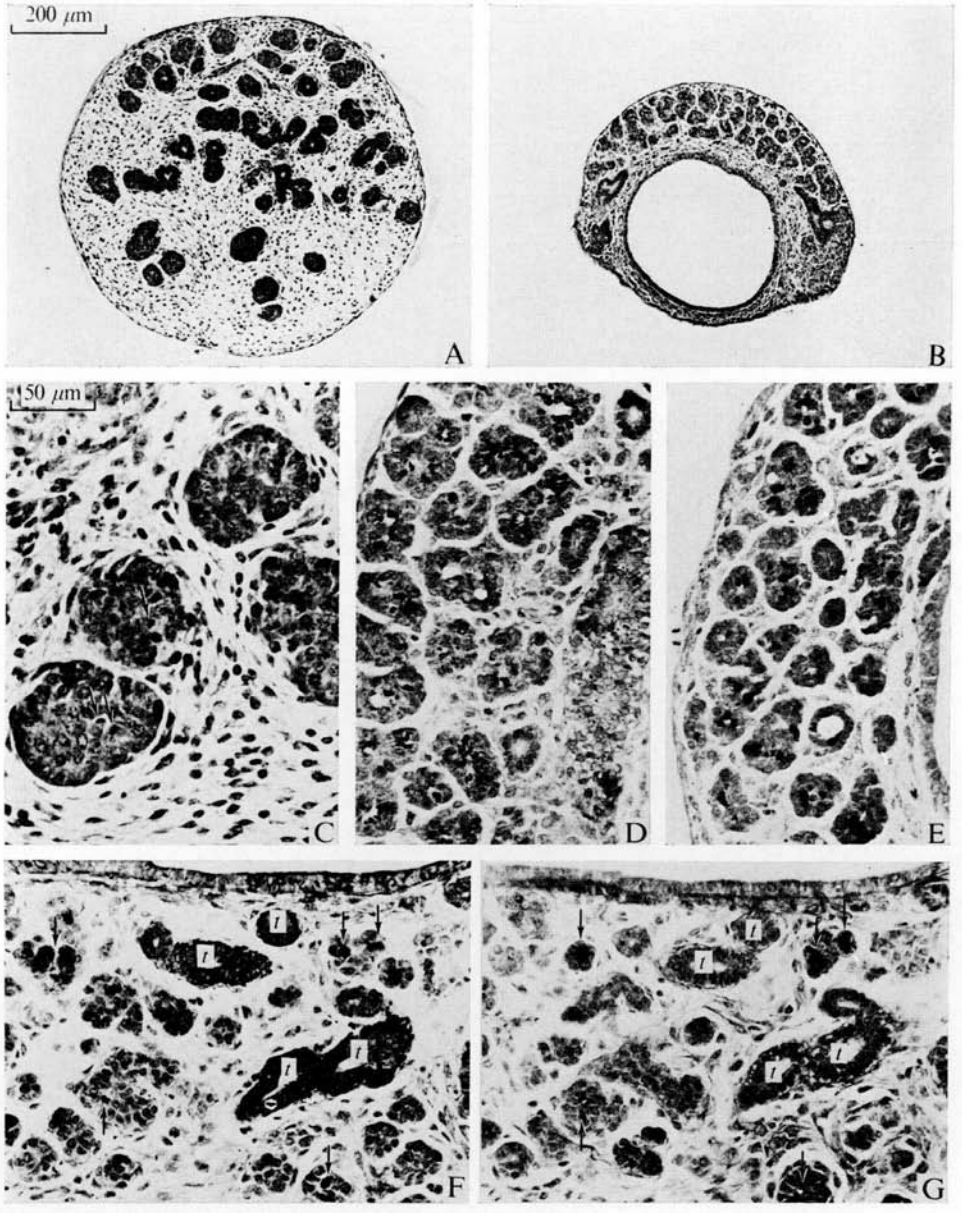


Fig. 11. Morphogenesis of mouse submandibular epithelium in its own mesenchyme (●), in 14-day rat lung mesenchyme (▲), and in 13-day rat lung mesenchyme (△). Vertical lines indicate the 95 % confidence limits for the means at any one time.

recombinate were used compared with two 14-day rat lung or two mouse submandibular mesenchymes. The experiment was set up in three replicates with three samples per treatment combination in each replicate; $N = 27$.

Even 13-day mesenchyme taken from round the primary bronchi (Fig. 1) was



able to support morphogenesis of mouse submandibular epithelium (Fig. 11), though the reinitiation of morphogenesis was slower than when mesenchyme from older embryos was used.

Effect of trypsin–pancreatin treatment

Although it is not possible to obtain whole epithelial rudiments free of mesenchyme without using enzyme treatment, substantial quantities of mesenchyme, from both submandibular and lung, can be obtained by dissection and

have been used by others in recombination experiments. Two series, trypsinized and dissected mesenchyme from 14-day mouse submandibular gland, 14-day rat lung and 12-day mouse lung were recombined with trypsinized mouse submandibular epithelium in a 2×3 factorial experiment in two replicates, with three samples per treatment combination in each replicate; $N = 36$.

It was found that trypsin–pancreatin treatment of the mesenchyme had no effect on the number of epithelial buds formed compared with dissected mesenchyme at any stage in any of the recombinates.

Differences between rat and mouse lung mesenchyme

Since rat and mouse lung mesenchyme are not quantitatively equivalent in their ability to support mouse submandibular morphogenesis, and since this difference becomes evident within 3 days of culture, early recombinates were examined histologically for (1) selective necrosis and (2) differences in distribution of acidic glycosaminoglycans, since such substances may play a key role in submandibular morphogeneis (Bernfield & Wessells, 1970; Bernfield & Banerjee, 1972; Bernfield, Banerjee & Cohn, 1972).

Necrotic areas were found within 18 h in lung mesenchyme from both mouse and rat in recombination with their own or mouse submandibular epithelium. Relatively more of the mouse than the rat mesenchyme appeared to be affected; the necrotic areas were not in contact with the epithelium, but peripheral to the blood vessels (Fig. 2F). Intact lungs that had been treated with trypsin–pancreatin, but not mechanically dissociated, also showed some necrosis in the mesenchyme which was more obvious in the mouse than in the rat material. Intact mouse lungs cultured without prior enzyme treatment occasionally had small necrotic areas; intact rat lungs were healthy. No such necrosis was found

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FIGURE 12

(A–E) Mouse submandibular epithelium in homo- and heterotypic recombination after 6 days culture: Helly-fixed, amylase-treated.

(A) With mouse submandibular mesenchyme.

(B) With rat lung mesenchyme. Magnification as in A.

(C) High-power view of explant shown in A. PAS-positive material indicated by arrows.

(D) High-power view of explant shown in B. Abundant PAS-positive material in buds. Magnification as in C.

(E) With 13-day mouse lung mesenchyme. Abundant PAS-positive material. Magnification as in C.

(F, G) Glycogen in heterotypic recombinate of rat parotid epithelium with lung mesenchyme cultured for 12 days. Carnoy fixation. Magnification as in C.

(F) No amylase treatment. Glycogen in tubules (*t*), amylase-resistant, PAS-positive material in buds (arrows). Compare with F.

(G) Amylase treatment. Section adjacent to F. Loss of PAS-positive material from tubules (*t*), retention in buds (arrows).

in submandibular mesenchyme. After 40 h culture necrotic patches were still present in the mesenchyme of recombinates of mouse submandibular epithelium with mouse lung mesenchyme, but not in any of the other recombinates. At 4 and 6 days the remaining mesenchyme cells appeared healthy.

Alcian blue-staining material with a critical electrolyte concentration of about 0·6 M-$MgCl_2$ was found at the epithelial–mesenchymal interface and intercellularly in the mesenchyme in all recombinates and intact explants at 18 h and subsequently. There was no obvious difference between any of the recombinates.

Effect of lung mesenchyme on histogenesis and cytodifferentiation of submandibular epithelium

Mouse submandibular epithelium had formed smaller, more closely packed buds after 5 days in lung mesenchyme than in its own mesenchyme (Fig. 12A, B). Such submandibular buds in lung mesenchyme were precociously differentiated, as shown by the presence of larger quantities of PAS-positive, amylase-resistant material compared with the homotypic controls (Fig. 12C–E). No such difference was seen in older '12-day' cultures (Fig. 2A–E).

Tubules formed by salivary epithelium in lung mesenchyme stained densely with PAS. Removal of this material with amylase indicates it to be glycogen (Fig. 12F, G). This effect of lung mesenchyme was particularly noticeable in parotid epithelium, since the tubules of the homotypic recombinates only occasionally contained traces of glycogen. The effect was least evident in recombinates with mouse submandibular epithelium since both buds and tubules in homotypic recombinates normally contained glycogen. Quantitative histochemistry has not been attempted.

DISCUSSION

The finding that lung mesenchyme is able to support morphogenesis and cytodifferentiation of rodent salivary epithelium *in vitro* contradicts previous reports (Grobstein, 1953; Spooner & Wessells, 1972) and makes untenable the hypothesis (Grobstein, 1967) that there is a mesenchyme-specific factor or property unique to salivary mesenchyme and essential for salivary morphogenesis. The conclusion applies to both submandibular and parotid epithelium from mouse and rat.

Since the direct contradiction was in the response of mouse submandibular epithelium to lung mesenchyme, the factors influencing tissue interaction in this recombinate were examined in more detail, using lung mesenchyme from both mouse and rat. A quantitative difference in the ability of submandibular and lung mesenchyme to support morphogenesis in submandibular epithelium was found: the smallest amount of submandibular mesenchyme tested – half that obtained from one 14-day submandibular gland – supported a normal rate of budding, whereas more than twice this mass of 12-day mouse lung

mesenchyme was required to evoke a morphogenetic response. Although no minimum effective mass of rat lung mesenchyme was demonstrated, both the number of buds and the volume of submandibular epithelium after 6 days culture were less than in the homotypic controls with the same initial amount of mesenchyme. The accompanying difference in the final number of mesenchyme cells between heterotypic and homotypic lung recombinates implies that lung epithelium is necessary for the maintenance and growth of lung mesenchyme and cannot be adequately replaced by submandibular epithelium. The relative amount of lung mesenchyme influences the growth of bronchial epithelium (Alescio, Cassini & Ladu, 1963; Alescio & Colombo Piperno, 1967; Alescio & Di Michele, 1968); a reciprocal effect of lung epithelium on the growth of lung mesenchyme has not been reported previously, although lung epithelium does influence the differentiation of lung mesenchyme (Taderera, 1967).

Such a requirement of lung mesenchyme for lung epithelium would account for the reduction in size with time of the heterotypic mouse recombinates and their improved development with larger initial masses of lung mesenchyme. Since the picture may be confused by selective necrosis in lung mesenchyme, the possibility of a direct and reciprocal relationship between epithelium and mesenchyme with respect to cell division deserves further investigation.

The quantitative difference between mouse lung and submandibular mesenchyme in evoking a morphogenetic response from submandibular epithelium could have contributed to Spooner & Wessells' (1972) negative results, but perhaps not to Grobstein's (1953): he used 6–8 pieces of mesenchyme of undefined size per recombinate. The nature of the non-nutrient substratum by which the explants were supported appears to have been the overriding factor: in the present experiments a mass of lung mesenchyme sufficient for supporting substantial submandibular growth and morphogenesis on an agar substratum evoked no, or only a meagre, response on MF. A further experiment with rat lung mesenchyme eliminated the nutrient medium as a source of the difference in response on the two substrata. The substrata used by Spooner & Wessells (MF) and by Grobstein (glass-clot interface) are alike in that they would be expected to encourage mesenchyme spreading. The agar substratum was originally chosen for salivary gland rudiments to repress this tendency in long-term cultures (Lawson, 1970). Mesenchyme spreading could lead to a reduction below the critical mass or critical density necessary for the interaction of a particular mesenchyme with submandibular epithelium. After 6 days culture there was no difference in the spacing of submandibular mesenchyme cells on agar or MF, nor in the number of epithelial buds formed in this mesenchyme on the two substrata; but lung mesenchyme cells, which were closely packed on an agar substratum, became dispersed on MF. This dispersion was greater in the heterotypic recombinates, accompanied by a reduction in bud formation. The slightly closer packing on MF of lung mesenchyme cells in association with their own as against submandibular epithelium could be expected from the

proposed growth-promoting action of lung epithelium on lung mesenchyme. If these observations are relevant to processes occurring earlier in the culture period, they imply that submandibular mesenchyme is effective at a lower cell density than lung mesenchyme in supporting branching morphogenesis.

It must be emphasized that the results under discussion concern the behaviour of lung mesenchyme that has been separated from the epithelium prior to recombination: the mesothelium investing the mesenchyme has therefore been extensively disrupted. The epithelium of the *intact* lung both grows and forms buds less rapidly on agar than directly on the surface of a plasma clot or on MF (unpublished observations).

Lung mesenchyme is not the only non-salivary mesenchyme able to support salivary morphogenesis: Cunha (1972) has shown that mouse submandibular epithelium will undergo extensive development in the mesenchyme of accessory sexual structures when the recombinates are cultivated in the anterior chamber of the eye of adult male mice. He suggests that sensitivity of mesenchyme to androgens is the characteristic determining whether a particular mesenchyme will support the development of salivary epithelium. If this explanation is appropriate for the interaction of submandibular epithelium with lung mesenchyme *in vitro* it must be assumed that (1) the 10% embryo extract and 10% foetal calf serum used in combination with Ham's F_{12} medium contained sufficient androgen to initiate the interaction (Eagle's basal medium plus 10% foetal calf serum contains no effective androgen (Cunha, 1973)); (2) F_{12} plus 10% foetal calf serum plus 10% embryo extract contains the same effective level of androgen as 66% cock plasma plus 33% embryo extract; (3) the interaction of lung epithelium with lung mesenchyme occurs via a different, non-androgen-sensitive mechanism. Alternatively, it is conceivable that foetal submandibular and lung mesenchyme can support the development of submandibular epithelium without mediation by androgens, whereas that from urogenital sinus or prostate can only do so in an androgen-containing environment. This does not necessarily imply that the basic mechanism is different.

A mechanism for salivary morphogenesis has been proposed (Bernfield & Wessells, 1970; Spooner & Wessells, 1970; Bernfield & Banerjee, 1972; Bernfield *et al.* 1972; Spooner & Wessells, 1972; Ash, Spooner & Wessells, 1973) in which epithelial clefts, which determine the branching points of the developing epithelial tree, are formed by the contraction of basal microfilaments in the epithelium. The conditions for cleft initiation are thought to be created by the deposition of proteoglycans and collagen at the epithelial–mesenchymal interface, for which mesenchyme is required. Lung epithelium characteristically does not form clefts, the change in contour of the epithelium at a branching point being much less sharp. It is noteworthy that submandibular epithelium, after initially forming large, flat buds in lung mesenchyme, then produces further buds peripherally by cleft formation. The materials present at the epithelial–mesenchymal interface in these recombinates, as well as in normal lung, clearly require further investigation.

The precocious appearance of PAS-positive material in submandibular buds formed in lung mesenchyme is accompanied by their closer packing and smaller size and the presence of a lumen. It is not known whether any of these factors are necessary or contingent for cytodifferentiation. The enhanced amount of glycogen, particularly in the tubular epithelium, is reminiscent of the large pools of glycogen normally present in morphogenetically active bronchial epithelium (Sorokin, Padykula & Herman, 1959; Sorokin, 1961; Alescio & Dani, 1971). However, there are reasons for thinking that the situation in the heterotypic recombinate is not analogous to that in lung, where the presence of epithelial glycogen is closely related to the morphogenetic activity of the epithelium: first, small amounts of glycogen are normally present in salivary tubular epithelium at some stages *in vitro*; secondly, the tubular epithelium is no longer forming new buds; thirdly, substantial quantities of glycogen were produced in the tubular epithelium of heterotypic recombinates cultured on MF, even when buds were few or absent. An alternative explanation is to suppose that salivary tubular epithelium will normally store glycogen unless restrained. Such a restraint could be provided by glycogenolytic agents in salivary mesenchyme, e.g. norepinephrine in association with the ganglion in the stalk region (Ash *et al.* 1973): nerve cells are present in cultured submandibular mesenchyme but have not been found in cultured foetal lung (J. Bluemink, personal communication). Another possibility is the hypothetical presence in salivary mesenchyme of special catecholamine-containing cells whose secretion could influence the neighbouring epithelium, as has been found in chick kidney mesenchyme cultured in combination with liver endoderm (Le Douarin & Houssaint, 1969; Le Douarin, 1971).

It is a pleasure to thank Miss B. M. van der Have for her skilled technical assistance, L. Boom for photographic work and Dr J. Faber for criticizing the manuscript.

Note added in proof: W. D. Ball (*J. exp. Zool.* (1974) **188**, 277–288) has reported that rat submandibular epithelium undergoes limited morphogenesis in lung mesenchyme when cultured at a plastic-clot interface. The results are similar to those described here for mouse submandibular epithelium in rat lung mesenchyme on MF. In contrast, in Ball's culture system the morphogenesis of rat sublingual epithelium in lung mesenchyme approached that of the controls.

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(*Received 14 February 1974*)