

Branching morphogenesis of mouse salivary epithelium in basement membrane-like substratum separated from mesenchyme by the membrane filter

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Summary

Branching morphogenesis of mouse salivary gland has been studied with organ-culture system. We developed a novel transfilter culture system for analyzing branching morphogenesis of the salivary epithelium. The submandibular salivary epithelium from early 13-day mouse fetus, clotted with Matrigel and separated from the mesenchyme by membrane filter, showed extensive growth and branching morphogenesis, morphological differentiation of lobules and stalks, and a typical cleft shape. The epithelium showed little growth and no branching without Matrigel clot or without the mesenchyme. This branching morphogenesis was induced even

when the pore size of the filter was reduced to 0.05 μm . Use of type I collagen gel instead of Matrigel mostly induced incomplete morphogenesis with various histological abnormalities. These results suggest that the salivary epithelium can undergo branching morphogenesis in the absence of the mechanical action of mesenchymal cells although it needs an appropriate extracellular matrix and some mesenchymal factors transmitted through the filter.

Key words: mouse salivary gland, branching morphogenesis, transfilter culture, Matrigel.

Introduction

Branching morphogenesis of mouse submandibular salivary gland has been well studied as an excellent model of embryonic organogenesis. Studies on epithelial–mesenchymal interactions revealed the important role of submandibular mesenchyme in the branching of submandibular epithelium. This morphogenesis of submandibular epithelium is supported by only a few kinds of heterotypic mesenchymes (Grobstein, 1953a; Cunha, 1972; Lawson, 1974). The submandibular mesenchyme can support branching morphogenesis of lung epithelium (Lawson, 1983), instructively influence mammary epithelium to branch in a salivary epithelium-like fashion (Kratochwil, 1969), and control lobular size of submandibular epithelium (Nogawa, 1983).

Extracellular matrices have been considered to be involved in the process of epithelial–mesenchymal interactions. In particular, collagens in the mesenchyme seem to have a crucial role in the branching morphogenesis of submandibular epithelium (Grobstein and Cohen, 1965; Spooner and Faubion, 1980; Nakanishi *et al.* 1986a,b,c; Fukuda *et al.* 1988). It is also known that fibroblastic cells possess collagen gel-contracting

activity (Bell *et al.* 1979; Stopak and Harris, 1982) and some authors suggested that cell traction of mesenchymal cells is involved in embryonic morphogenesis and pattern formation (Oster *et al.* 1983). Nakanishi *et al.* (1986c) suggested a model for the branching morphogenesis of the submandibular epithelium in which the traction force generated by the mesenchymal cells *via* collagen bundles deforms the epithelial surface and results in cleft formation. In respect of the force of submandibular mesenchyme, Nogawa and Nakanishi (1987) reported that it was able to move plastic beads by its flowing movement *in vitro*, in addition to its ability to bring about collagen-gel contraction.

In an earlier study, Grobstein (1953b) found a 'branching' structure with clefts formed by submandibular epithelium clotted with rooster plasma on a membrane filter, with its mesenchyme below the filter. Although the epithelial morphology differed from the typical branching pattern of submandibular epithelium, Grobstein's work has shown that epithelial morphogenesis can be partly induced in the absence of the mechanical action of the mesenchyme.

In the present study, we tried to analyze the morphogenetic behaviour of the submandibular epithelium outside the mechanical action of the mesen-

chyme by transfilter experiments. During the course of this study, it was found that the epithelium, when separated by membrane filter from the mesenchyme, did not show branching morphogenesis, probably because the extracellular environment of the epithelium was quite different from that *in situ*. In order to provide the epithelium with the appropriate extracellular environment, we clotted it with extracellular matrix. Matrigel, a reconstituted matrix of basement membrane components from EHS sarcoma (Kleinman *et al.* 1986), is known to promote differentiation of various epithelial cells (Hadley *et al.* 1985; Kubota *et al.* 1988). We used Matrigel and collagen gel for clotting the epithelium, and found remarkable morphogenetic behaviour of the submandibular epithelium.

Materials and methods

Preparation of tissues

ICR mice were mated during the night, and the day of discovery of the vaginal plug was designated as day 0. Rudiments of submandibular gland were dissected from early 13-day fetuses in Hanks' balanced salt solution (HBSS). The stalk region was removed from the rudiment, and the lobular epithelium and surrounding mesenchyme were treated with dispase (1000 protease units ml^{-1} in HBSS; Godo Shusei Co., Tokyo, Japan) at 37°C for 30 min. After being washed three times in HBSS, epithelium and mesenchyme were separated with fine forceps. The separated tissue fragments were then stored in HBSS with 20% horse serum (Gibco Lab.) at room temperature and used in the following experiments.

Transfilter experiments

Details of the original transfilter technique are described by Grobstein (1956), while our method differs from his in some respects. Isopore membranes (pore size 0.4 μm , thickness 10 μm , Millipore Co., Bedford, MA) and Nuclepore filters (pore size 0.4, 0.1 and 0.05 μm , thickness 10, 5 and 5 μm , respectively, Nuclepore Co., Pleasanton, CA) were used in the transfilter experiments. Both kinds of filters are made of polycarbonate and have straight pores made by irradiation of charged particles. The membrane filter was autoclaved, cut into small pieces and stored in HBSS with 20% horse serum before use. Three pieces of mesenchymal fragments were assembled and used with one isolated lobular epithelium per explant.

The experimental procedure is illustrated schematically in Fig. 1. Four kinds of explants were made. (1) The epithelium was directly recombined with the mesenchyme on membrane filter. (2) The epithelium was placed on the opposite side of the filter to the mesenchyme. (3) The epithelium was separated by the filter from the mesenchyme as in the second case, and was clotted with Matrigel (Collaborative Research Inc., Bedford, MA). (4) Only the epithelium was clotted with Matrigel on the filter, without the mesenchyme. Matrigel was dropped on the epithelium from the tip of a cooled tuberculin syringe, and was allowed to gel at 37°C.

We also used collagen gel (Cellmatrix type I-A: acid-soluble fraction of type I collagen from porcine tendon, Nitta Gelatine Co., Osaka, Japan) instead of Matrigel in later experiments. A mixture of Cellmatrix type I-A, 10 \times medium 199 Earle's BSS (Gibco Lab.) and 200 mM Hepes buffer solution (8:1:1, by vol.) was made on ice and was used like Matrigel. The final concentration of collagen was 2.4 mg ml^{-1} .

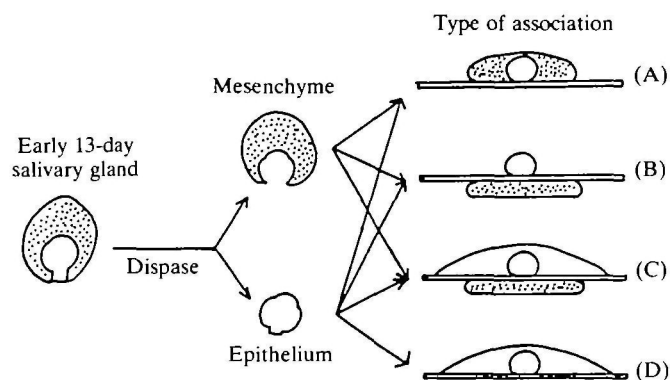


Fig. 1. Procedure of the transfilter experiments. (A) Direct recombine. (B) Transfilter recombine without Matrigel clot. (C) Transfilter recombine with Matrigel clot. (D) The epithelium only with Matrigel clot on the filter.

All the explants were cultivated on semisolid medium composed of medium 199 Earle's BSS with 20% horse serum and 0.5% agar (Difco Lab.) at 37°C in 5% CO_2 in air. Living explants were photographed before fixation.

Histology

Explants were fixed in 10% neutral formalin overnight or in Bouin's solution for 3 h. The samples were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections of 5 μm were made, stained with hematoxylin and eosin, and observed with a light microscope.

Results

Transfilter experiments using Matrigel

The submandibular epithelium from the early 13-day fetus is a spherical cell mass with no definite clefts on its surface but sometimes showing a few shallow clefts forming (Fig. 2). After cultivation for 3 days, the epithelium that had directly recombined with the mesenchyme showed extensive growth and typical

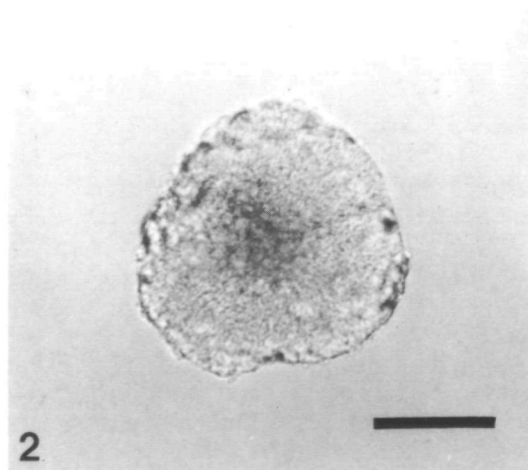


Fig. 2. Early 13-day submandibular epithelium isolated from the mesenchyme. No definite cleft is seen on the epithelial surface. Bar, 100 μm .

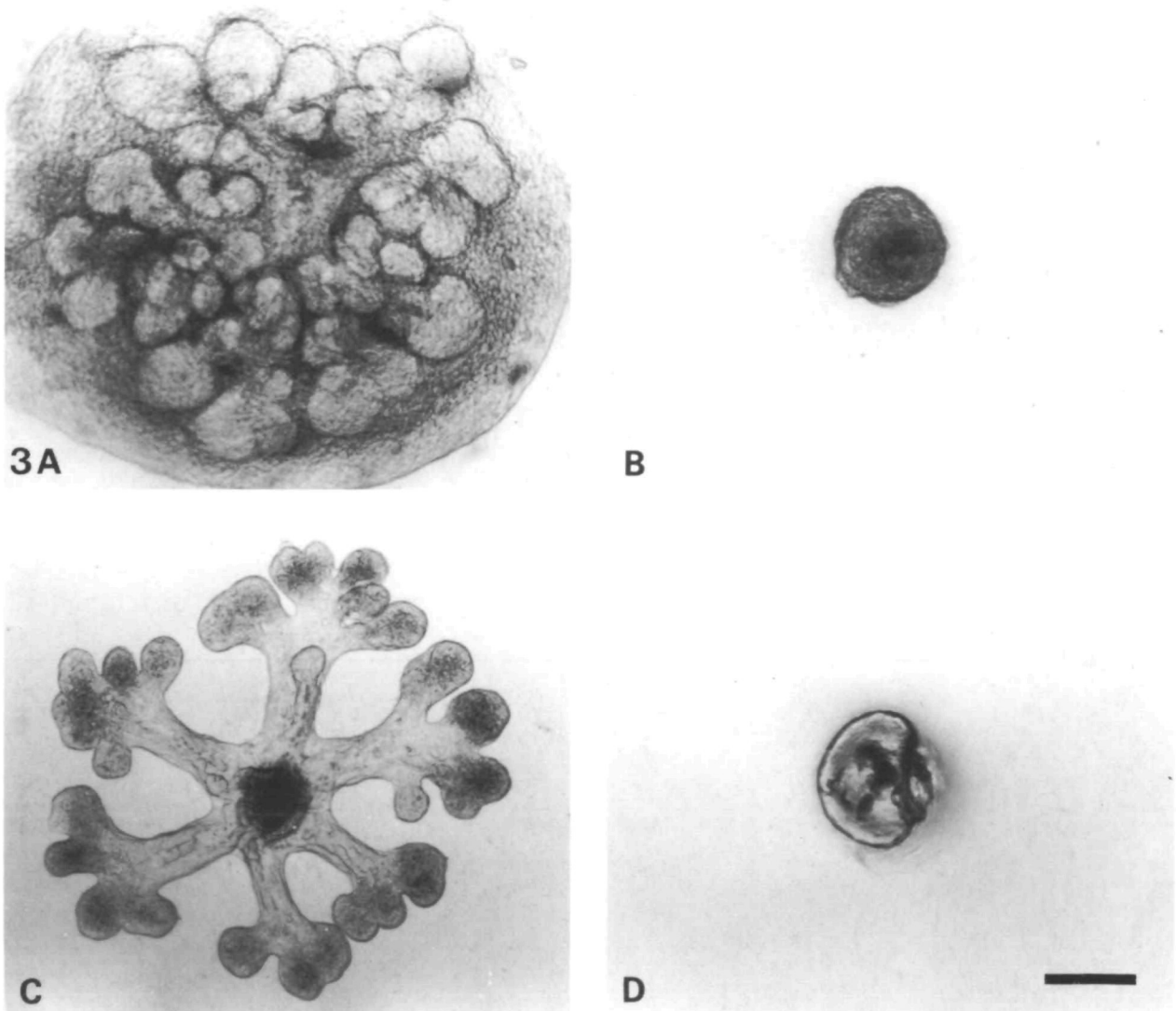


Fig. 3. Results of the transfilter experiments with Matrigel. (A) The epithelium directly recombined with the mesenchyme on the filter and cultivated for 3 days. Extensive growth and typical branching morphogenesis of the epithelium are seen. (B) The epithelium separated from the mesenchyme by the filter and cultivated for 3 days. Little growth and no branching morphogenesis are seen. (C) The epithelium clotted with Matrigel and separated from the mesenchyme by the filter and cultivated for 3 days. The epithelium shows extensive growth and branching morphogenesis. Notice the morphological differentiation of lobular and stalk regions. (D) The epithelium clotted with Matrigel without the mesenchyme and cultivated for 3 days. The epithelium shows little growth and no branching, but formation of a cyst with a lumen. Living explants were photographed after the filters (A,D) or the filters and the mesenchymes (B,C) were removed. Bar, 200 μm .

branching morphogenesis (Fig. 3A) as well as an intact rudiment in organ culture. The epithelium that was separated from the mesenchyme by the filter (Isopore, pore size 0.4 μm) showed little growth and no branching in any of the 20 cases (Fig. 3B). In contrast, all the epithelia clotted with Matrigel and separated from the mesenchyme by the filter showed extensive growth and almost typical branching morphogenesis (Fig. 3C) like the epithelium that had directly recombined with the mesenchyme. The most remarkable and characteristic feature of this epithelium is the morphological differentiation of the lobular and stalk regions. This epithelium, however, grew flatter in shape as a whole and had a smaller number of lobules than the epithelium directly

recombined with the mesenchyme (Table 1). On the other hand, the epithelium clotted with Matrigel and cultured without mesenchyme showed no substantial growth and no branching in any of the 24 cases. This epithelium formed a simple small cyst containing fluid in its lumen (Fig. 3D). The epithelium also showed no branching when only the mesenchymal part was clotted with Matrigel in the transfilter culture.

Examination of the time course of the branching morphogenesis of the epithelium clotted with Matrigel and separated from the mesenchyme by the filter revealed that the mode of cleft formation at the lobules shows a remarkable resemblance to that in the epithelium directly recombined with the mesenchyme

Table 1. Branching morphogenesis of the submandibular epithelium in transfilter experiments using Matrigel

Explants	Culture period (days)	No. of lobules per explant*
Direct recombine	3	40.0±9.8 (10)
Transfilter recombine with Matrigel clot	1	5.5±0.7 (10)
	2	8.3±2.9 (13)
	2.5	14.9±4.1 (13) UC†: 3/16
	3	21.1±8.4 (49) UC: 14/63

* Mean±s.d. (no. of explants).

† Uncountable due to epithelial fusion (see Fig. 4E).

(Fig. 4, Table 1). One day after explantation, definite deep clefts were seen on the epithelial cell mass, thereby dividing it into several lobules (Fig. 4A). At 2 days, the lobules extended radially and some of them had new clefts on their expanded distal tips, their proximal parts forming stalks (Fig. 4B,C). At 3 days,

further growth and cleft formation resulted in an increase in the number of the lobules and extension of the stalks (Fig. 4D). The tips of the epithelium often extended over the boundary of the mesenchyme. Budding was occasionally observed at the lateral side of the stalks, but was not observed in the epithelium directly recombined with the mesenchyme. In some explants, the lobules began to fuse with each other laterally at 2.5 days and, furthermore, they had fused too much to count the lobule number in about 20 % of the explants at 3 days (Table 1, Fig. 4E).

Histological observation also confirmed the similarity between the structure of the epithelium branching in Matrigel and branching in the mesenchyme (Fig. 5A,B,C). In both cases, a lumen was formed in the central part of the epithelium and extended radially into the stalks. The cells lining the lumen were columnar and some cell debris was seen in the lumen. The distal lobules were epithelial cell masses without lumina, where somewhat columnar cells were located at the basal layer.

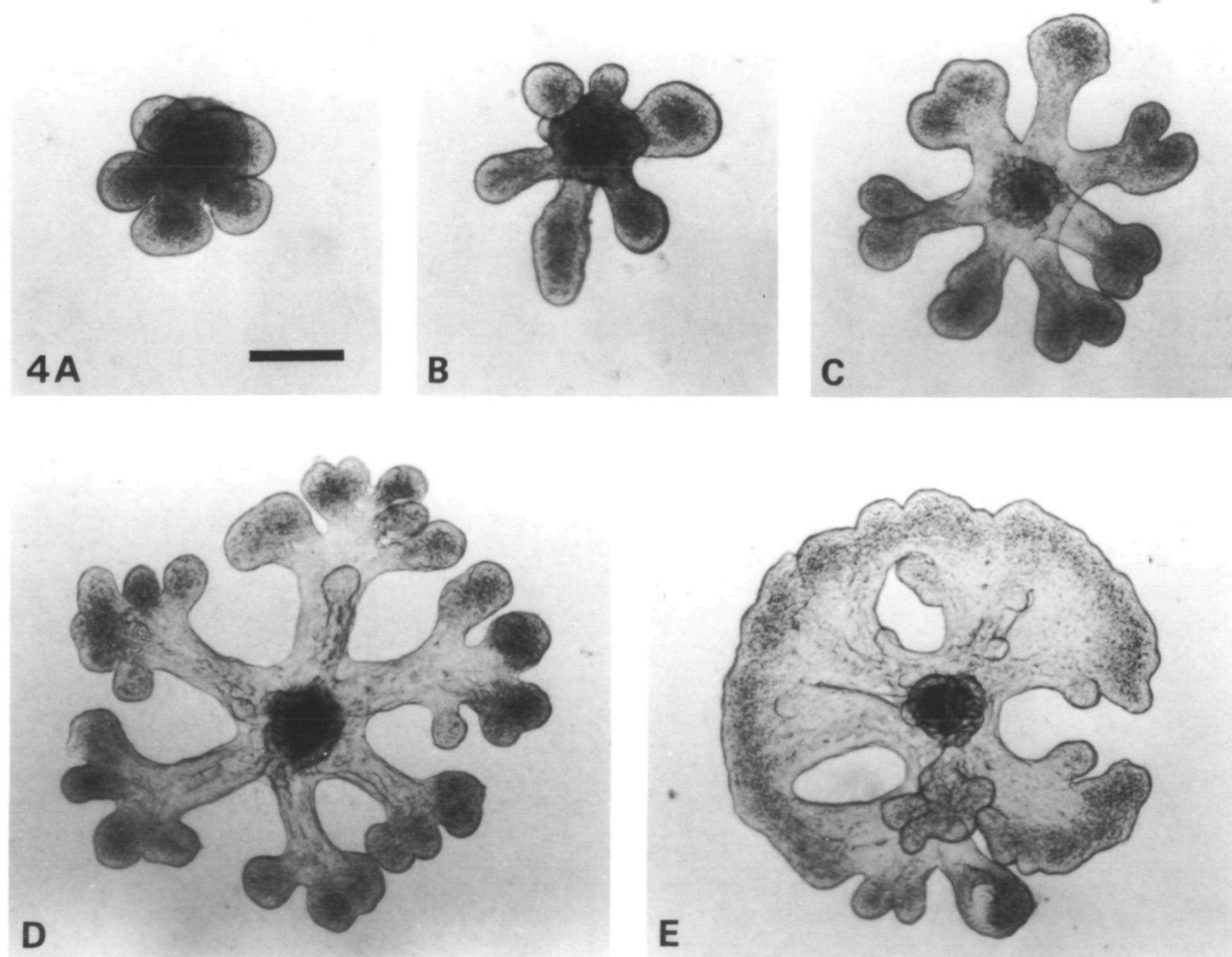


Fig. 4. Time course of the branching morphogenesis of the epithelium clotted with Matrigel and separated from the mesenchyme by the filter. (A) 1 day; (B) Early 2 day; (C) Late 2 day; (D) 3 day after explantation. (E) One of the epithelia at 3 day with extreme fusion of neighbouring lobules. Bar, 200 μ m.

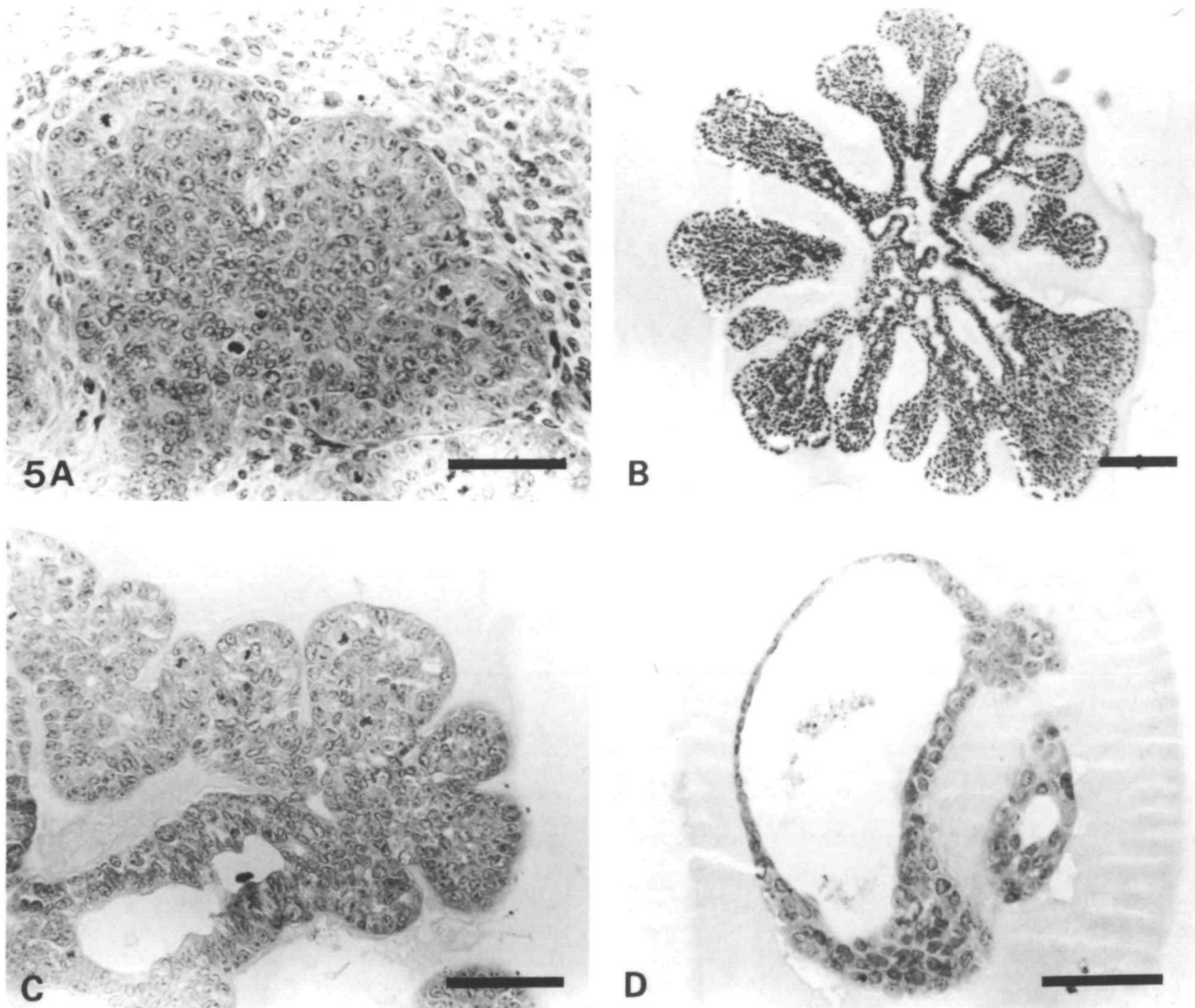


Fig. 5. Sections of the explants in the transfilter experiments using Matrigel. (A) The direct recombine. (B) The branching epithelium in Matrigel with the mesenchyme below the filter. The lumen extends into the stalks. (C) Higher magnification of the same type of recombine with (B). The lobules are epithelial cell masses with somewhat columnar cells at the basal layer. The lumen is lined with columnar cells. (D) The epithelium clotted with Matrigel without the mesenchyme showing cyst formation. The lumen filled with fluid and some cell debris is lined with often simple epithelial layer. Bars, (A,C,D) 50 μm . (B) 100 μm .

The epithelium clotted with Matrigel and cultured without mesenchyme formed a single cyst with a large lumen filled with fluid. In this case, the epithelium around the lumen consisted of a simple cuboidal or squamous cell layer (Fig. 5D). The epithelium separated from the mesenchyme by the filter without clotting became a spherical cell mass without lumen and was sometimes necrotic.

Detailed observation of serial sections of the explants revealed that there were no mesenchymal cells around the epithelium of transfilter explants.

Effects of various pore sizes of the filter

In order to obtain information about mesenchymal influence through the filter, we used Nuclepore filters with smaller pore sizes. The epithelium was clotted with

Matrigel, separated from the mesenchyme by a Nuclepore filter of pore size 0.4, 0.1 or 0.05 μm and cultivated. Each example showed extensive growth and branching morphogenesis, and there was statistically no significant difference in the number of lobules among the cases with different pore sizes, though total pore area per filter is smaller in the filter with smaller pore size (Fig. 6, Table 2).

Transfilter experiments using collagen gel

We used collagen gel instead of Matrigel in the transfilter experiments to determine if there was a difference in the epithelial response to the different kinds of extracellular matrices. The epithelium clotted with collagen gel and separated from the mesenchyme by the filter (Isopore, pore size 0.4 μm) showed various

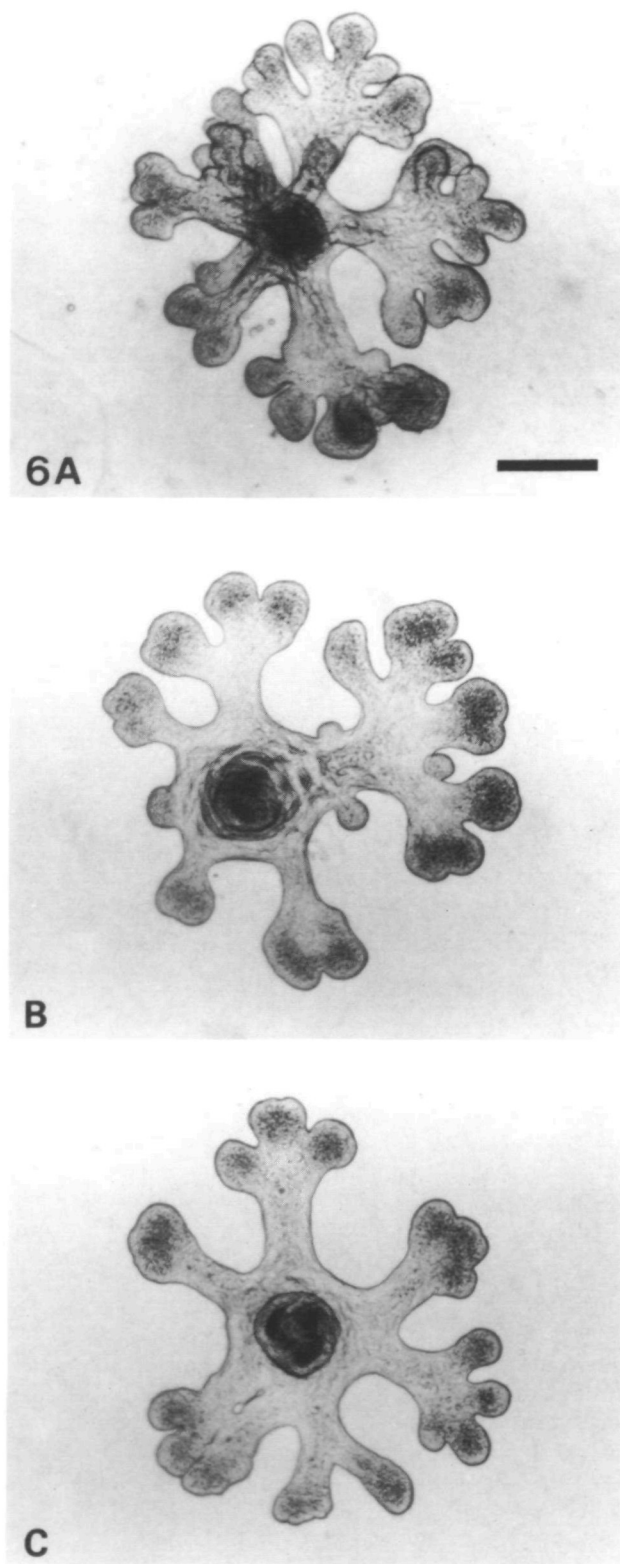


Fig. 6. Branching morphogenesis of the epithelium clotted with Matrigel and separated from the mesenchyme by the filters with various pore sizes. (A) 0.4 μm . (B) 0.1 μm . (C) 0.05 μm . Bar, 200 μm .

Table 2. Effects of various pore sizes of the filter on branching of the epithelium in transfilter experiments

Pore size (μm)	Total pore area ($\text{mm}^2\text{cm}^{-2}$)	No. of lobules per explant*
0.4	12.56	18.1 ± 7.4 (20)
0.1	2.36	15.5 ± 8.4 (21)
0.05	1.18	14.3 ± 7.6 (21)

* Mean \pm S.D. (no. of explants) at 3 days of cultivation.

responses when cultured (Fig. 7, Table 3). Some remained as a spherical cell mass and did not branch at all. Most of the explants showed abnormal morphogenesis, as follows. In some explants, the epithelium formed several protrusions and so had an undulating outline but no sharp clefts (Fig. 7A). In others, fairly normal clefts and lobules were formed, but the epithelial cells dissociated from the tissue (Fig. 7B). In others, the epithelium formed small satellite-like cell masses around it, probably caused by separation of epithelial buds from the central part of the epithelium (Fig. 7C,D). Only one out of 30 explants showed normal cleft formation but no stalk formation.

The epithelium clotted with collagen gel without mesenchyme showed little growth and no branching, with a rough surface and some extent of cell dissociation (Fig. 7E).

Histologically, the incompletely branching epithelium in collagen gel formed a lumen in its center and showed generally similar architecture to the branching epithelium in Matrigel, although the former had some abnormal features in tissue organization as stated above. Frequently, small lumina were observed near the basal surface in the lobules of the incompletely branching epithelium (Fig. 7F). These were rarely seen in the epithelium directly recombined with mesenchyme or in the branching epithelium in Matrigel.

Discussion

Branching morphogenesis of the fetal mouse submandibular gland has been studied mainly from the point of view of epithelial–mesenchymal interaction. The role of cell proliferation, cell shape change and extracellular matrices in branching morphogenesis has been investigated to date, but these studies were based on experiments with an organ-culture system, in which the two kinds of tissues, the epithelium and the mesenchyme, interacted as easily as *in situ*. A more analytical experimental system needed to be introduced for further study of the epithelial–mesenchymal interactions in this organ.

In the present study, we found that the submandibular epithelium clotted with Matrigel and separated from the mesenchyme by a filter showed extensive branching morphogenesis with a remarkable resemblance to that of the normally branching epithelium. Grobstein (1953b) carried out transfilter experiments and found ‘branching’ morphogenesis of the submandibular epi-

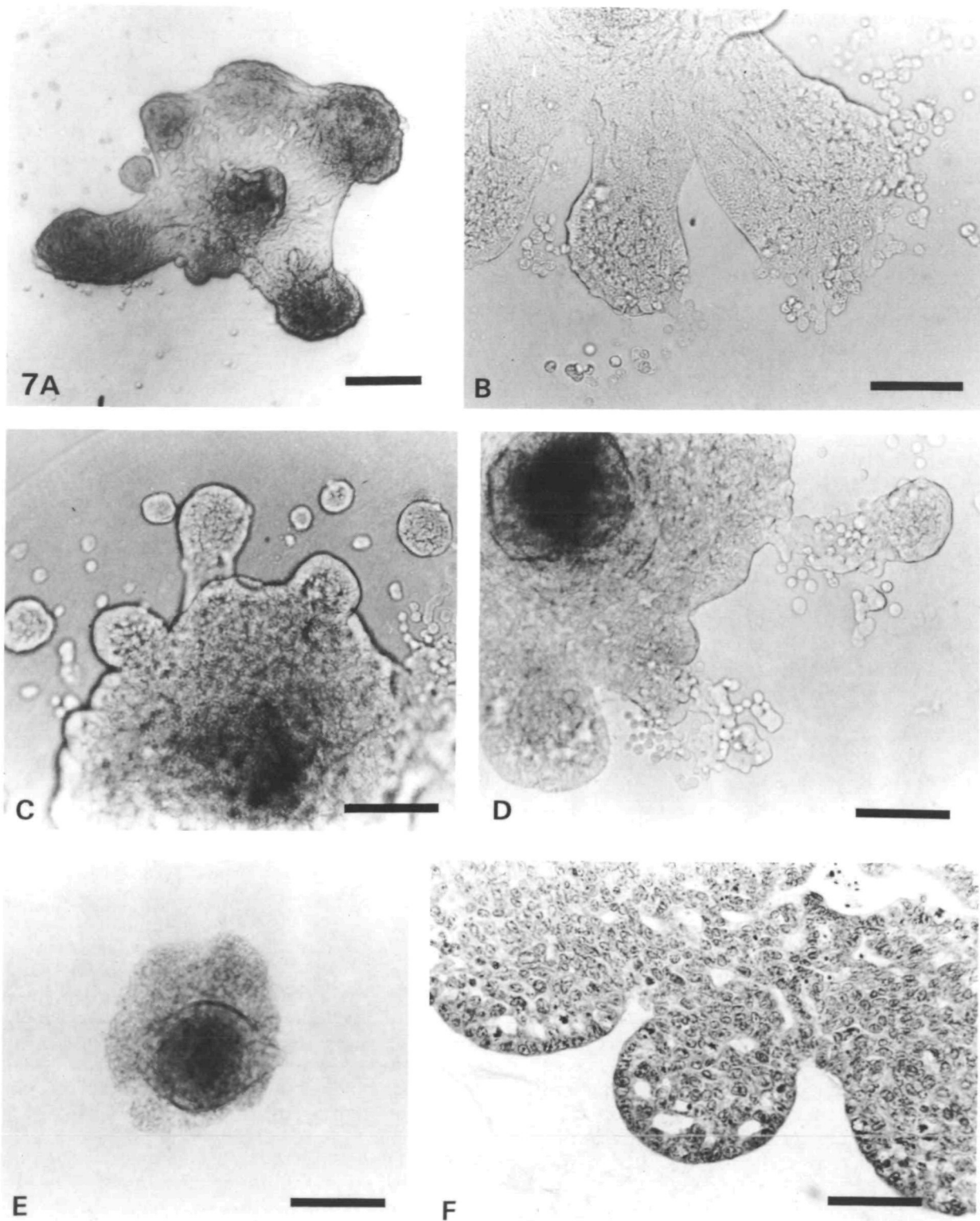


Fig. 7. Morphology of the epithelium in the experiments using collagen gel. (A–D) The epithelia clotted with collagen gel and separated from the mesenchyme by the filter and cultivated for 3 days. Incomplete or abnormal branching patterns are seen. (A) One of the epithelia with several protrusions but without typical sharp clefts. (B) One with fairly normal lobular shape but with dissociation of epithelial cells. (C) One with small satellite-like cell masses around itself. (D) The satellite-like cell masses are formed probably by separation of epithelial buddings. (E) The epithelium clotted with collagen gel without the mesenchyme and cultivated for 3 days. Little growth and no branching are seen. (F) A section of the incompletely branching epithelium in collagen gel. Small lumina are seen beneath the basal layer. Bars: (A) 200 μm , (B–E) 100 μm , (F) 50 μm .

Table 3. Morphology of the submandibular epithelium in the transfilter experiments using collagen gel instead of Matrigel

Explants	Culture period (days)	No. of explants with branching			
		None	Abnormal	Normal	(Total)
Clotted with collagen gel	2	3	4	0	(7)
and separated from mesenchyme by the filter	3	3	26	1	(30)
Clotted with collagen gel without mesenchyme	3	7	0	0	(7)

thelium clotted with plasma and separated from the mesenchyme by a membrane filter. However, the epithelial shape, with very deep clefts on its surface, was different from the typical shape in intact organ culture. He called it 'atypical morphogenesis' and offered no explanation of the difference in the epithelial form. Grobstein used a mixture of rooster plasma, embryo extract and horse serum as the clotting substance, probably to fix the epithelium to the membrane filter rather than to provide appropriate conditions for the epithelium. The difference between Grobstein's and our results seems to reflect the difference in the components of the clotting substances.

The present transfilter system enabled us to analyse the action of the mesenchyme on the epithelium, including the effects of some extracellular matrices, diffusible factors, direct cell-to-cell contact, and the mechanical action of the mesenchymal cells such as cell movement and traction force. The model described by Nakanishi *et al.* (1986c) attributed cleft formation on the epithelial surface to the traction force generated by the mesenchymal cells *via* collagen bundles. But this model seems to overestimate the role of the mechanical action of the mesenchyme and now should be questioned, since the shape of the branching epithelium without any mesenchymal cells around it in the present study is almost identical to that observed in the normally branching gland. The generation of the fundamental morphogenetic force should be assigned to the epithelial side, although the mechanical action of the mesenchyme seems to play some role in the branching morphogenesis, since Nogawa and Nakanishi (1987) reported that the plastic-beads-moving activity of the mesenchyme from various organs was correlated with branching-inducing activity.

In the present study, the submandibular epithelium showed fair growth and branching morphogenesis when clotted with Matrigel and separated from the mesenchyme by a filter of pore size 0.4, 0.1 or 0.05 μm . In kidney development, which has been well analyzed by transfilter experiments, metanephric tubule induction is correlated with direct cell-cell contact between two tissues by the penetration of mesenchymal cell processes through the filter. When submandibular mesenchyme was used as a heterotypic inducer, penetration of cell processes was hardly observed in the Nuclepore filter of pore size 0.6 μm , and was never observed in the Nuclepore filter of pore size 0.2 μm (Saxen, 1980). Comparing these data with our results, the mesenchymal action through the filter on branching morphogen-

esis of the submandibular epithelium in the present study appears to be mediated by some diffusible factors.

The epithelium clotted with Matrigel or collagen gel and separated from the mesenchyme by the filter grew far better than the epithelium separated from the mesenchyme by the filter without clotting. This means that the gels of extracellular matrices provide a good extracellular environment for epithelial growth. Similarly, the epithelium clotted with Matrigel or collagen gel with mesenchyme on the opposite side of the filter showed far better growth than the one without mesenchyme. This obviously indicates that mesenchymal factors through the filter stimulate epithelial growth. Cell proliferation is probably necessary for extensive branching morphogenesis, but stimulation of epithelial growth is not enough to induce branching morphogenesis, because most epithelia clotted with collagen gel and separated from the mesenchyme by a filter showed reasonable growth but no typical branching.

Type I collagen gel is a widely used substratum useful for epithelial tissue culture, and permits some kind of morphogenetic behaviour of epithelial cells from several organs. However, the submandibular epithelium did not show normal morphogenesis in collagen gel in the present transfilter system. The abnormal features induced in the epithelia in collagen gel, such as dissociation of cells from the epithelial tissue and separation of satellite-like cell masses, suggest that these epithelia lacked the appropriate regulation of cell-cell adhesion that is required to maintain the stable and unified organization of epithelial cells. The frequent formation of small lumina beneath the surface layer shows random expression of cell polarity, and may cause the collapse of the epithelial tissue.

The most characteristic feature of the branching epithelium in Matrigel is the morphological differentiation of lobules and stalks. The time course study of the branching process of this epithelium showed somewhat precocious differentiation of stalks. In fact, some explants at day 3 had very long stalks with a small number of lobules. Considering that the differentiation of lobules and stalks was never observed in the incompletely branched epithelia in collagen gel, the basement membrane components contained in Matrigel seem to play an important role in stalk formation as well as in typical cleft formation at the expanding lobules. The epithelium clotted with Matrigel without mesenchyme formed a cyst with a thin epithelial layer

surrounding the lumen. This also suggests that the basement membrane components permit some kind of reorganization of epithelial cells.

The major components of basement membrane and Matrigel are laminin, type IV collagen, heparan sulfate proteoglycan, nidogen and entactin (Kleinman *et al.* 1986). The important role of basal lamina proteoglycan in maintaining lobular morphology and in branching morphogenesis was suggested by Bernfield and his group (Bernfield *et al.* 1972; Banerjee *et al.* 1977). Laminin was recently reported to be involved in the expression of epithelial cell polarity in metanephric tubule formation (Klein *et al.* 1988), endothelial cell alignment in formation of capillary-like structure (Grant *et al.* 1989), and normal branching of lung epithelium (Schuger *et al.* 1990). These studies suggest that laminin should be included with other basement membrane components as a candidate with activity of epithelial cell organization. The effects of each basement membrane component on the morphogenetic behaviour of the submandibular epithelium need to be investigated in future studies.

The authors thank Professor K. Shiokawa, Dr S. Yasugi and Dr H. Fukamachi of University of Tokyo for their advice and encouragement during the course of this work, and especially Dr Yasugi for his helpful comments on the manuscript.

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(Accepted 2 November 1990)