

## Epithelial induction of osteogenesis in embryonic chick mandibular mesenchyme studied by transfilter tissue recombinations

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### SUMMARY

The initiation of osteogenesis in the mandibular mesenchyme of the embryonic chick at 7 days is dependent upon an epithelial induction which occurs in the mandible up to the fourth day *in ovo*. In the present study, transfilter tissue recombinations were used to study this inductive mechanism. The epithelial and mesenchymal components of the mandibles were separated before the completion of the induction and recombined to form transfilter explants which were either cultured for 9 days or grafted onto the chorioallantoic membrane for host embryos for 7 days.

Control experiments demonstrated that the tissue separation and recombination techniques did not interfere with the normal epithelial induction, and confirmed that mandibular mesenchyme isolated at this stage was incapable of forming bone.

Bone was observed in 86 % of the CAM-grafted intact mandible controls and in 80 % of the cultured intact mandible controls. Bone failed to form in the mesenchyme of transfilter explants when Millipore filters with 0.45  $\mu\text{m}$  pores were used. Bone was observed as frequently as in control explants when the mandibular mesenchyme was separated from its epithelium by 0.8  $\mu\text{m}$  or 0.4  $\mu\text{m}$  porosity Nuclepore filters. Only about 30 % of the transfilter explants prepared with 0.1  $\mu\text{m}$  porosity Nuclepore filters formed bone and none of the explants prepared with 0.03  $\mu\text{m}$  porosity Nuclepore filters formed bone. SEM studies demonstrated a distinct correlation between the formation of bone in transfilter explants and the ability of the epithelium and mesenchyme to penetrate the pores of the filters.

Thus, the present study provides evidence that the site of the induction is restricted to the epithelial–mesenchymal interface, and that the induction is not mediated by a diffusible substance. The nature of the inductive mechanism is discussed with respect to this and other recent studies which suggest that the induction may be mediated by a non-diffusible epithelial cell product resident in the epithelial basal lamina.

### INTRODUCTION

Cells derived from the cranial neural crest migrate into the mandibular arch of the embryonic chick during the second and third days of development (Le Lievre and Le Douarin, 1975). This population of cells forms an ectomesenchymal mass which ultimately differentiates into the bone, cartilage, endothelial

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smooth muscle and the dermal portion of the feathers of the mandibular process (Le Lievre, 1978; Nodan, 1978). The mandibular ectomesenchyme requires a permissive inductive interaction with the mandibular epithelium until Hamburger & Hamilton (1951) stage 24 ( $4\frac{1}{2}$  days) in order to begin to form bone at H. H. stage 33 ( $7\frac{1}{2}$  to 8 days) (Tyler & Hall, 1977). Inductive interactions have been shown to be similarly prerequisite to osteogenesis in the maxillary, palatine, scleral and cranial skeletons of the embryonic chick (Schowing, 1968; Hall, 1978*a*, 1981*a*; Tyler, 1980, 1981; Tyler & McCobb, 1980), and in the mandible and calvarium of the embryonic mouse (Hall, 1980*b,c*). The inductive interactions involved in skeletogenesis have recently been reviewed by Hall (1980*b,c*, 1981*b*, 1983*a-c*).

Previous experiments on the inductive interaction required for the initiation of osteogenesis in the chick mandibular process have revealed that 1) the epithelium must be alive and mitotically active (Hall, 1980*a,b*), 2) the inductive competence of the epithelium is not tissue specific (Hall, 1978*b*) or species specific (Hall, 1980*b*); and 3) chemical suppression of epithelial collagen and/or glycosaminoglycans renders the epithelium inductively inactive (Bradamante & Hall, 1980). This final observation led us to investigate whether the induction was mediated by an extracellular cell product. Two series of experiments were undertaken to address this question. The first was to determine whether extracellular products of cultured epithelial cells contain inductive activity which they do (Hall & Van Exan, 1982). The second, reported here, was to determine whether or not the inductive action was mediated through a diffusible substance. To achieve this, mandibular mesenchyme, isolated from its epithelium at a stage prior to the completion of induction, was cultured or grafted transfilter to its inductively active epithelium. The transfilter technique, where a permeable membrane is interposed between the putative inducer and the responding tissue, was devised by Grobstein (1953, 1956) and has been successfully applied to the analysis of the development of the nervous system, eye, kidney, teeth and cartilage (see Wessells, 1977, Sawyer & Fallon, 1983 and Hall, 1983*b* for reviews).

## MATERIALS AND METHODS

### *Incubation procedure*

Fertile eggs of the domestic fowl, *Gallus domesticus*, White Leghorn, Shaver starcross 288 strain, were obtained from Cook's Hatchery, Truro, Nova Scotia. The eggs were incubated without rotation in a forced-air, Humidaire Incubator (Model 350, Humidaire Incubator Co., New Madison, Ohio, U.S.A.) maintained at  $37 \pm 0.5^\circ\text{C}$  and  $57 \pm 4\%$  relative humidity.

### *Tissue preparation*

The eggs were incubated for 95 h and opened under sterile conditions. The embryos were removed and placed in sterile saline (0.85% NaCl) for staging

according to the morphological series of H. H. stage described by Hamburger & Hamilton (1951). Only H. H. stage-22 embryos were used in this study.

The mandibular processes were carefully dissected from the embryos and an equal number were placed in each of the following two sterile solutions: 1) trypsin/pancreatin solution (257 mg beef pancreas trypsin and 43 mg porcine pancreatin/10 ml Ca, Mg-free Tyrode solution) for 1 h at 4°C, or 2) BGJb culture medium with 15 % horse serum for 1 h at room temperature. The enzymes were obtained from BDH Chemicals, Toronto, Ontario, Canada and the culture medium from GIBCO (Canada), Burlington, Ontario, Canada.

#### *Control experiments*

The following series of control experiments were conducted to determine the effects of the tissue separation technique on the epithelial induction of bone in the mandibular mesenchyme. Intact mandibles from solution 2 (not enzyme treated) were cultured or CAM-grafted in host embryos. Some of the mandibles treated with enzymes in solution 1 were placed directly in culture. Other enzyme treated mandibles were separated into their epithelial and mesenchymal components in a mixture of BGJb and horse serum (1:1 v/v) and subsequently recombined with no interposing filter before being CAM-grafted into host embryos. The epithelium was removed from a third group of enzyme treated mandibles and the remaining mesenchyme was either cultured or CAM-grafted in the absence of epithelium.

#### *Transfilter experiments*

The study of membrane bone induction in the chick mandible by transfilter tissue recombination posed certain problems not encountered in the study of other inductive interactions by the same method. The time between induction and osteogenesis is unusually long. Bone is first detectable 9 days after induction in culture, 7 days after induction in CAM-grafts and 3 days after induction *in vivo* (Hall, 1978). In transfilter experiments, the isolated epithelium, though healthy and intact at the beginning of the culture period when the induction occurs, could not always be found when bone was detectable in the mesenchyme 9 days later. This is because the epithelium is normally maintained through contact with the adjacent mesenchyme (Tyler & Hall, 1977). Failure of bone to form in a mandible could be because the filter blocked the induction or because early degeneration or movement of the epithelium removed it before the inductive interaction occurred. To solve these problems the procedure shown in Fig. 1 was developed. Briefly, an intact mandible (not treated with enzyme) was placed epithelial side down on a Millipore support filter. The epithelium was peeled off an enzyme-treated mandible and placed over the mesenchymal surface of the intact mandible. A test filter was then placed over the epithelium and the remaining piece of isolated mesenchyme was placed on the test filter in direct apposition to the underlying epithelium. The mesenchyme of the intact mandible both

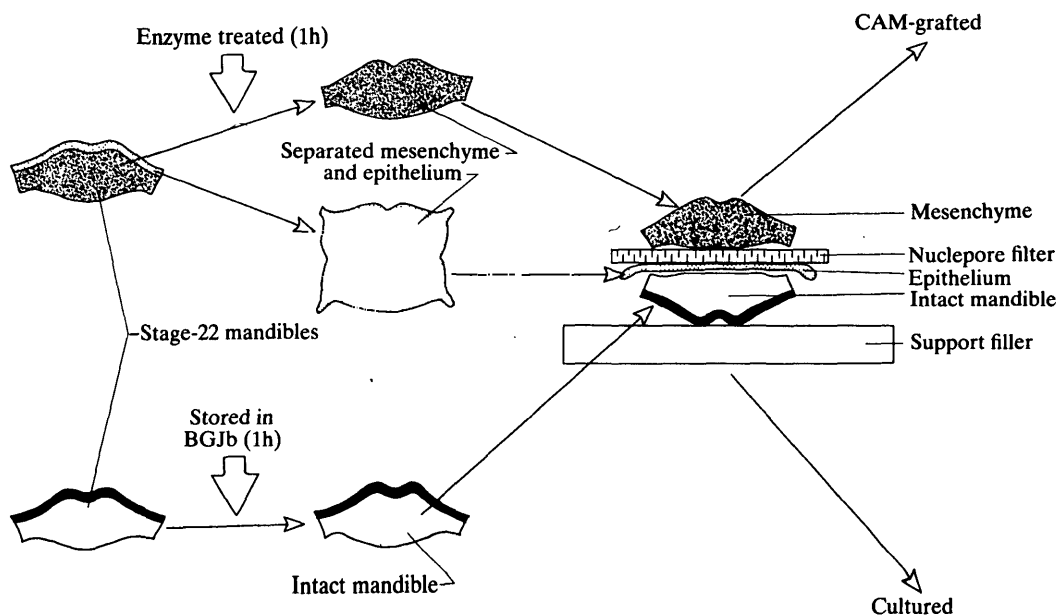


Fig. 1. This diagram shows the method of preparing transfilter explants. Explants prepared in this manner were either placed on a metal grid support filter side down and cultured for 9 days or placed mesenchyme side down onto the chorioallantoic membrane of a host embryo.

served to support the healthy growth and differentiation of the isolated epithelium during culture and provided a control for mandibular bone formation in an intact mandibles exposed to the same environment as the transfilter recombination. A further control set of transfilter recombinations between mesenchyme from mandibles of H. H. stages 22 and 24 was used to show that the mesenchyme itself lacked inductive activity. Any induction transfilter could therefore be attributed to epithelial action. The isolated mesenchyme was separated from its epithelium by one of the following test filters: 1) HABP Millipore filter, 150  $\mu\text{m}$  thick, 0.45  $\mu\text{m}$  pore diameter, 2) THWP Millipore filter, 25  $\mu\text{m}$  thick, 0.45  $\mu\text{m}$  pore diameter, 3) N080 Nucleopore filter, 10  $\mu\text{m}$  thick, 0.8  $\mu\text{m}$  pore diameter, 4) N040 Nucleopore filter, 10  $\mu\text{m}$  thick, 0.4  $\mu\text{m}$  pore diameter, 5) N010 Nucleopore filter, 10  $\mu\text{m}$  thick, 0.1  $\mu\text{m}$  pore diameter, or 6) N003 Nucleopore filter, 5  $\mu\text{m}$  thick, 0.03  $\mu\text{m}$  pore diameter. Millipore filters were obtained from the Millipore Filter Corp., Bedford, Mass., U.S.A. and Nucleopore filters were obtained from the Nucleopore Co., Pleasanton, CA., U.S.A.

Transfilter preparations were either cultured for 9 days or CAM-grafted in host embryos for 7 days as described below.

#### *Culture and CAM-grafting procedures*

Cultured explants were placed on stainless steel grids in Falcon plastic Petri

dishes containing 1.5 ml of BGJb with 15 % horse serum and 150 µg/ml ascorbic acid (Matheson, Coleman & Bell, Norwood, Ohio, U.S.A.). The cultures were maintained at 37°C in a humidified CO<sub>2</sub> incubator (Forma Scientific, Model 3156) in an atmosphere of 5 % CO<sub>2</sub> in air. The medium was completely changed every three days.

CAM-grafted explants were placed on the vascularized chorioallantoic membrane of 8-day host embryos through a window cut in the shell. The window was sealed with tape and the host with graft was incubated at 37°C (Hall, 1978c).

#### *Histological procedures*

Grafts and cultures were fixed for 24 h in neutral buffered formal saline. Tissues were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin. Serial 6 µm sections were cut and stained according to a modification of Lison's procedure with haematoxylin, alcian blue and chlorantine fast red. Sections were examined for the presence or absence of bone and cartilage and for the status of the epithelium.

#### *Scanning electron microscopy*

Experiments were conducted to determine whether epithelial and mesenchymal cell processes could penetrate any of the test filters used in the transfilter experiments. In these experiments, epithelia were separated from stage-22 mandibles and placed on test filters. The filters with the epithelium on the top surface, were placed over mandibular mesenchyme and cultured for 24 h as described above. The filters were processed for scanning electron microscopy to examine both epithelial and mesenchymal surfaces. Filters were dehydrated in graded acetones and critical-point dried in a Sorvall drying system (Ivan Sorvall Inc., Nortown, Connecticut). The dried filters were placed on stubs and grounded with silver. Subsequently, filters were coated with a thin layer of gold palladium under vacuum in a Nonotech SEM Prep 2 Sputter Coater (Nono Tech Ltd., Manchester). Stubs were examined in a Cambridge S1-50 SEM.

## RESULTS

### *Control experiments*

The results of control experiments are summarized in Table 1. Bone was observed in 86 % of intact mandibles after 7 days in CAM-graft and in 80 % of intact mandibles after 9 days in culture. These data were obtained from observation of individual explants of intact mandible and from intact mandible controls maintained with transfilter preparations (a total of 65 CAM-grafts and 51 cultures). Membrane bone was found in 89 % of the intact mandibles which had been treated with enzyme for 1 h and cultured for 9 days. Membrane bone was also present in 85 % of mandibles which had been separated into their epithelial

Table 1. *Results of control experiments of intact, enzyme-treated, recombinations and isolated mesenchyme*

TISSUE*	CAM-GRAFTS			CULTURES		
	n	% cart	% bone	n	% cart	% bone
Intact mandible	65	100 %	86 %	51	100 %	80 %
Enzyme-treated mandible	—	—	—	9	100 %	89 %
Recombined mandible	7	100 %	85 %	—	—	—
Mesenchyme alone	10	100 %	0 %	15	100 %	0 %

\* All tissues form H. H. Stage-22 chick embryos, CAM-grafted for 7 days or cultured for 9 days.

and mesenchymal components and recombined without an interposing filter prior to CAM-grafting for 7 days. These observations indicate that the experimental procedures involved in separating the mandibular epithelium from its mesenchyme did not interfere with the ability of the epithelium to induce bone or with the ability of the mesenchyme to respond to the induction by forming bone. None of the CAM-grafts or cultures of mandibular mesenchyme contained membrane bone indicating that H. H. stage-22 mesenchyme requires an epithelial induction for bone differentiation to occur (Table 1).

#### *Transfilter experiments*

Serial sections of transfilter explants stained with alcian blue to demonstrate cartilage and with chlorantine fast red to demonstrate bone, were examined microscopically. The intact mandibular portion of each transfilter explant, whether cultured for 9 days or CAM-grafted for 7 days, possessed a central rod of cartilage (Figs 2–5). This indicated that the explanted mandibular processes

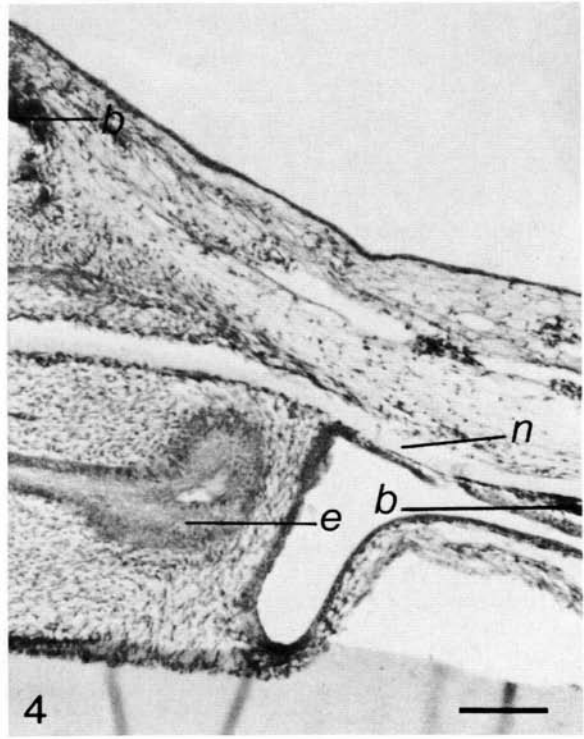
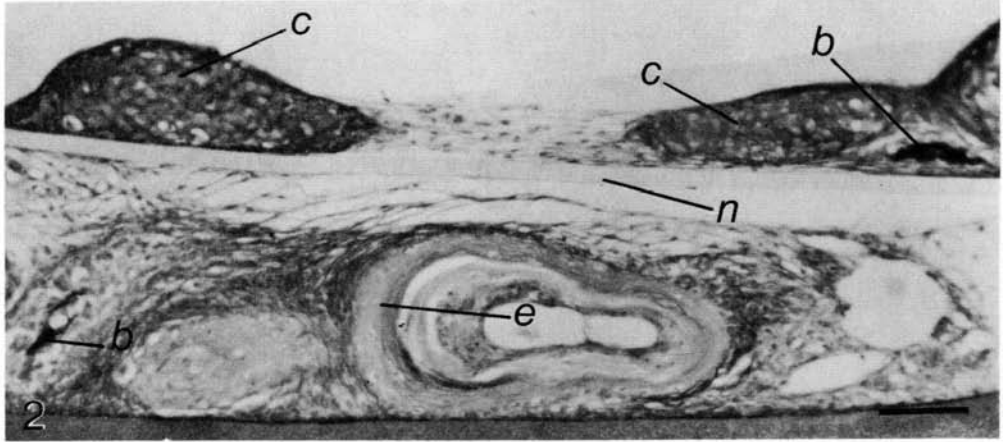
Fig. 2. Transfilter explant prepared with a  $0.8 \mu\text{m}$  Nuclepore filter (n) and cultured for 9 days. The intact mandible portion of the explant below the filter contains healthy epithelium (e), cartilage (not shown in this section) and bone (b). The isolated mesenchyme above the filter contains cartilage (c) and bone (b) but no epithelium. Bar =  $30 \mu\text{m}$ .

Fig. 3. A transfilter explant prepared with a  $0.4 \mu\text{m}$  pore diameter Nuclepore filter between the intact mandible and mesenchymal portions. The explant was CAM-grafted for 7 days. The intact mandible contains epithelium (e), cartilage (c) and bone (b). Above the Nuclepore filter (n) the isolated mesenchyme contains cartilage (c) and bone (b) but no epithelium. Bar =  $60 \mu\text{m}$ .

Fig. 4. This transfilter explant was prepared with a  $0.1 \mu\text{m}$  pore diameter Nuclepore filter and was CAM-grafted for 7 days. The intact mandible portion below the Nuclepore filter (n) contains epithelium (e), cartilage (not shown in this section) and bone (b). Bone (b) was also observed above the filter in the isolated mesenchymal portion of this explant. Bar =  $50 \mu\text{m}$ .

were healthy and that the culture and grafting techniques supported tissue differentiation. Bone was usually observed near the cartilage in the intact mandibular portions of cultures or grafts (Figs 2-6). The presence of bone in the intact mandible portion of a transfilter explant demonstrated that the culture or grafting technique supported osteogenesis.

The epithelium in cultured transfilter explants usually remained in proximity



Figs 2-4

to the isolated mesenchyme located on the opposite side of the filter. However, after 9 days in culture, the epithelium was curled into a hollow sphere which was keratinized on the inside (Figs 2, 5). Likewise, in CAM-grafted explants, the epithelium usually remained in place directly beneath the isolated mesenchyme on the opposite side of the filter. However, the epithelium formed a large ball or isolated islets after 7 days incubation (Figs 3, 4, 6).

The isolated mesenchymal portion of the transfilter explants usually remained in position directly across the filter from its epithelium. A central rod of cartilage was observed in the isolated mesenchyme but these tissues were completely free of epithelium (Figs 2–6).

The presence or absence of membrane bone in the isolated mesenchyme was related to the type of filter placed between the mesenchyme and its epithelium as is demonstrated below. The location of the membrane bone, when present in the isolated mesenchyme, was the same as that observed in the intact mandible, close to the cartilage rod but not necessarily close to the inductively active epithelium on the other side of the filter. The percentage of transfilter explants in which membrane bone was observed in the isolated mesenchyme is shown in Fig. 7. Only explants meeting the following conditions were used to tabulate these data: 1) the explants had to be healthy as indicated by the presence of cartilage in both the isolated mesenchyme and in the intact mandible portions of the explant, 2) the mesenchymal portion of the explant had to be completely free of mandibular epithelium and 3) the mandibular epithelium had to be healthy and located directly beneath its mesenchyme on the other side of the interposing filter (i.e. explants in which the epithelium or mesenchyme had moved out of position during the culture or graft period were not used in the tabulation of these results). Bone was observed in the *intact* mandibular portion of 80 % of the 59 transfilter explants cultured for 9 days. The intact mandible of transfilter explants CAM-grafted for 7 days contained membrane bone in 86 % of the 78 grafted explants. These data are summarized in Fig. 7.

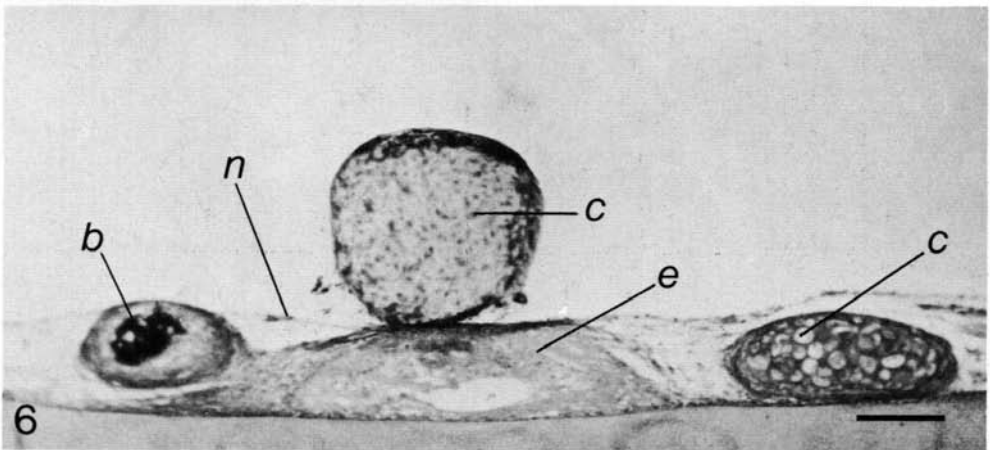
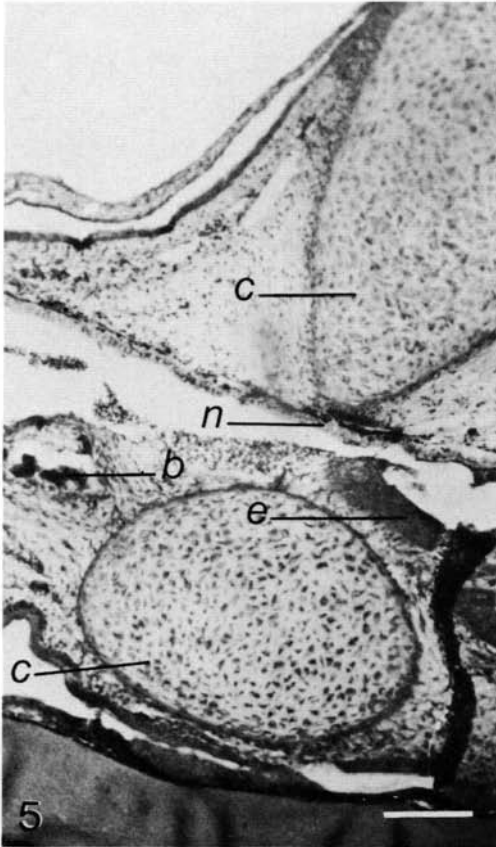
When Millipore filters (0.45  $\mu\text{m}$  pore size) were placed between the mandibular mesenchyme and its epithelium, the induction was blocked and membrane bone failed to develop in the mesenchyme. Epithelial induction of mandibular bone

Fig. 5. The transfilter explant shown here was prepared exactly as that shown in Fig. 4 with a 0.1  $\mu\text{m}$  pore diameter Nuclepore filter. The intact mandible portion below the filter (n) contains epithelium (e), cartilage (c) and bone (b). The isolated mesenchyme above the filter contains healthy cartilage but no epithelium or bone. Bone was induced in the isolated mesenchyme of only 33 % of the transfilter explants prepared with 0.1  $\mu\text{m}$  Nuclepore filters (Fig. 7). Bar = 50  $\mu\text{m}$ .

Fig. 6. This transfilter explant was prepared with a nuclepore filter of 0.03  $\mu\text{m}$  pore diameter and was cultured for 9 days. The intact mandible portion below the filter (n) contains epithelium (e), cartilage (c) and bone (b) while the isolated mesenchyme consists primarily of cartilage (c) and contains no bone or epithelium. Induction of bone formation in the isolated mesenchyme of explants prepared with this type of filter was blocked in 100 % of the explants examined (Fig. 7). Bar = 30  $\mu\text{m}$ .



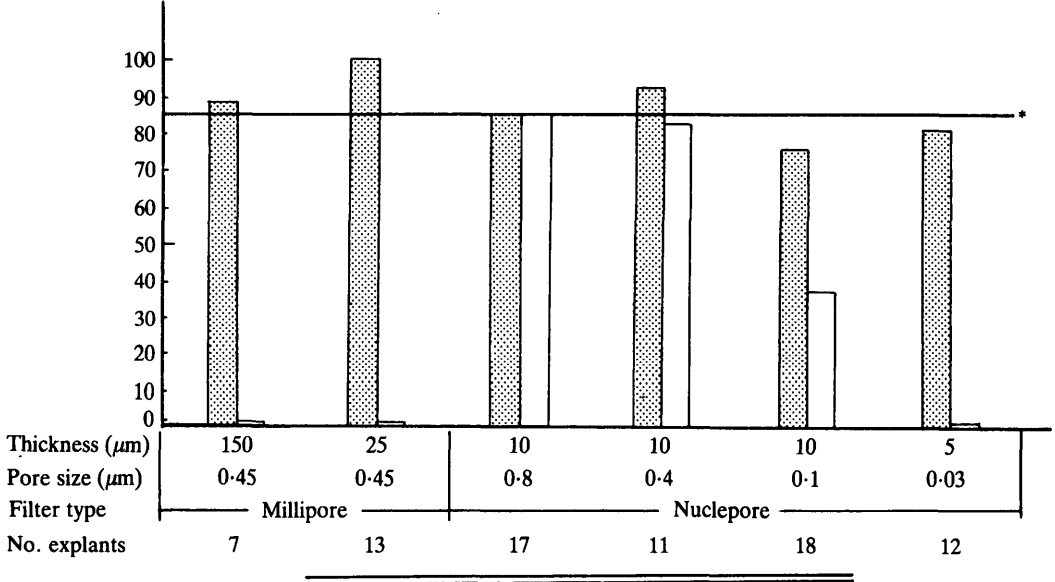
was transmitted through Nucleopore filters of  $0.8\ \mu\text{m}$  and  $0.4\ \mu\text{m}$  pore diameter since bone was observed in the isolated mesenchyme about as frequently as it was in the intact mandible of these explants (Figs 2, 3, 7). Membrane bone was



Figs 5 & 6

- Stage-22 mesenchyme transfer to epithelium
- ▨ Intact stage-22 mandible control
- \* Average for all controls

A: CAM GRAFTS



B: CULTURES

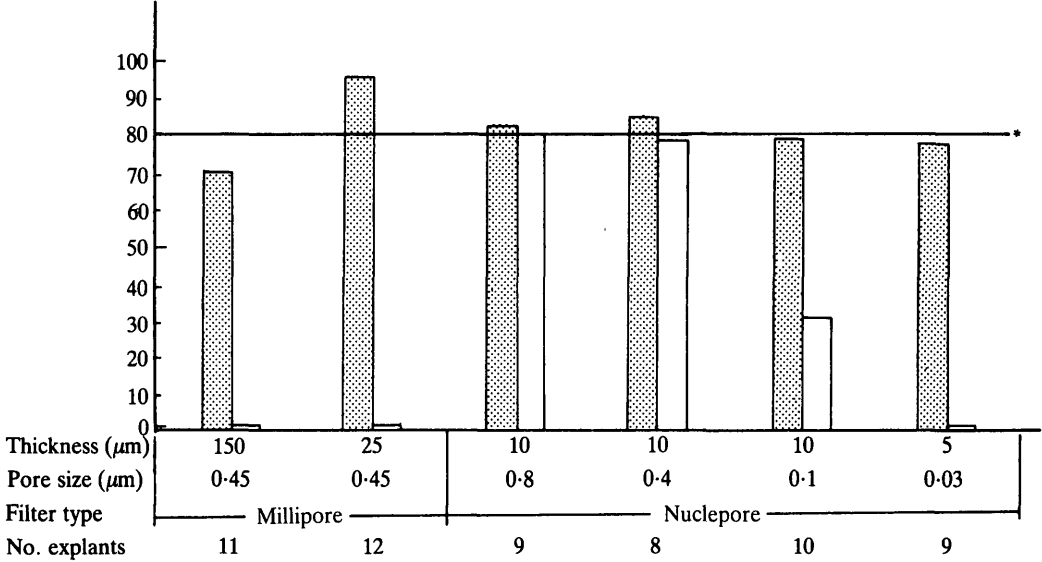


Fig. 7

observed in the isolated mesenchyme of only 33 % of the explants prepared with Nuclepore filters of  $0.1 \mu\text{m}$  pore diameter (Figs 4, 5), a percentage significantly lower than that observed in the intact mandible controls of the same transfilter explants (Fig. 7). When transfilter explants were prepared with Nuclepore filters of  $0.03 \mu\text{m}$  pore diameter, no bone formed in the isolated mesenchyme (Figs 6, 7), indicating that these very thin ( $5 \mu\text{m}$ ) filters could block the inductive action of the epithelium. To confirm that epithelium and not mesenchyme of the intact mandibles located transfilter to the isolated mesenchyme was responsible for inducing bone in the isolated mesenchyme, recombinations of H. H. stage-24 mandibular mesenchyme were established transfilter to H. H. stage-22 mesenchyme, using filters of  $0.4 \mu\text{m}$  porosity. Mesenchyme from H. H. stage-24 embryos is able to form bone when isolated – the induction is completed by then (Tyler & Hall, 1977). It was reasoned that if mesenchyme accumulated active epithelial inducer with time, H. H. stage-24 mesenchyme would have done so and would have been able to induce H. H. stage-22 mesenchyme to form bone. No bone formed in the H. H. stage-22 mesenchyme so cultivated while bone did form in the already-induced H. H. stage-24 mesenchyme maintained transfilter to it (4/4 cases). We concluded that transfilter inductive activity could not be attributed to the mesenchyme of the intact mandibles.

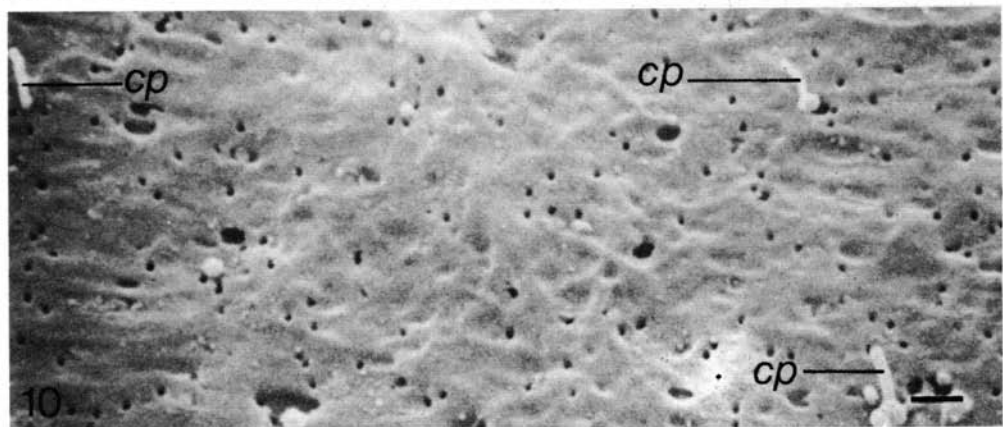
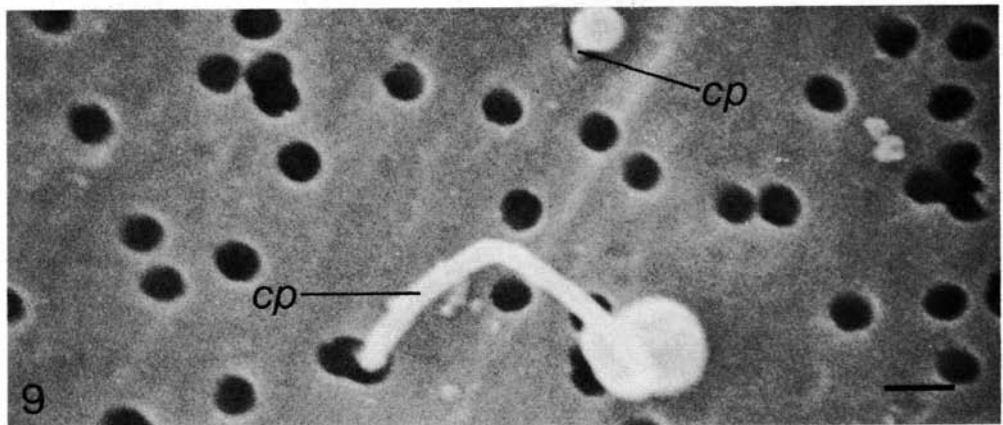
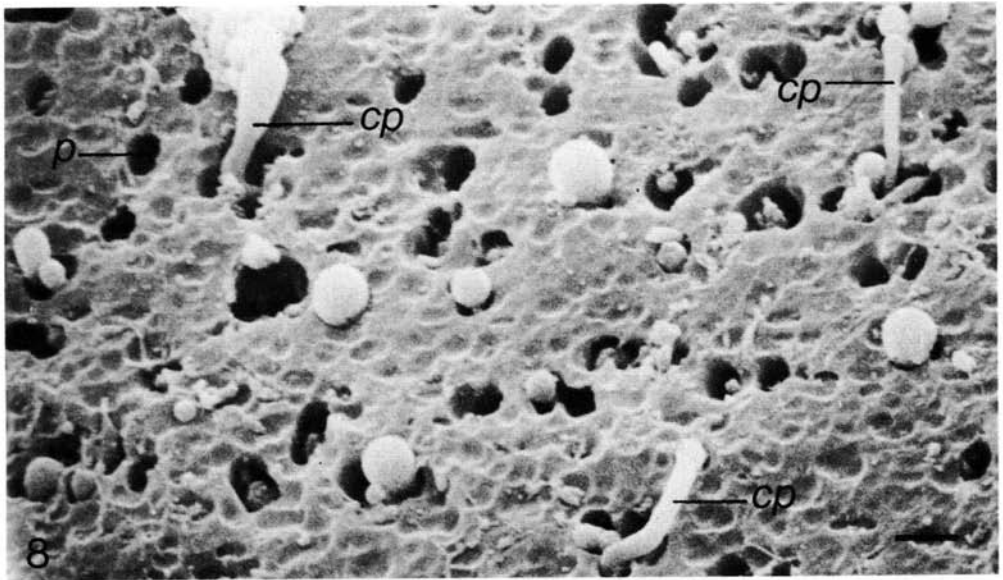
#### *Scanning electron microscopy*

Epithelial cell processes were observed penetrating all three of the  $0.8 \mu\text{m}$  pore size Nuclepore filters on which mandibular epithelium had been cultured transfilter to mesenchyme for 24 h (Fig. 8). The processes ranged from  $0.25$  to  $0.5 \mu\text{m}$  in diameter, averaged  $0.35 \mu\text{m}$  in diameter, were observed over a large area of each filter and were densely packed. Similar observations were made on Nuclepore filters of  $0.4 \mu\text{m}$  pore size (Fig. 9). Although processes were observed penetrating an extensive area of all three filters examined, the density of the processes per unit area was less than that observed in the  $0.8 \mu\text{m}$  pore size filters and the average diameter ( $0.22 \mu\text{m}$ ) was somewhat smaller. Only one of the three  $0.1 \mu\text{m}$  pore size filters was penetrated by epithelial cell processes, and these were present only in a small area of the filter and at a relatively low density (Fig. 10). The average diameter of the protruding cell processes ( $0.1 \mu\text{m}$ ) was the same size or slightly larger than the diameter of the pores through which they passed. Cell processes were never observed penetrating Nuclepore filters of  $0.03 \mu\text{m}$  pore diameter.

Mesenchymal cell processes exhibited a similar pattern, penetrating the pores

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Fig. 7. The percent bone formation in transfilter explants CAM-grafted for 7 days or cultured for 9 days is summarized in this histogram. There was no correlation between the ability to induce bone in the isolated mesenchyme with either the filter thickness, or the filter type. However, among the nuclepore filters tested, there was a decrease in the percent bone formation in the mesenchyme with correspondingly smaller pore diameters in the filters.



Figs 8-10

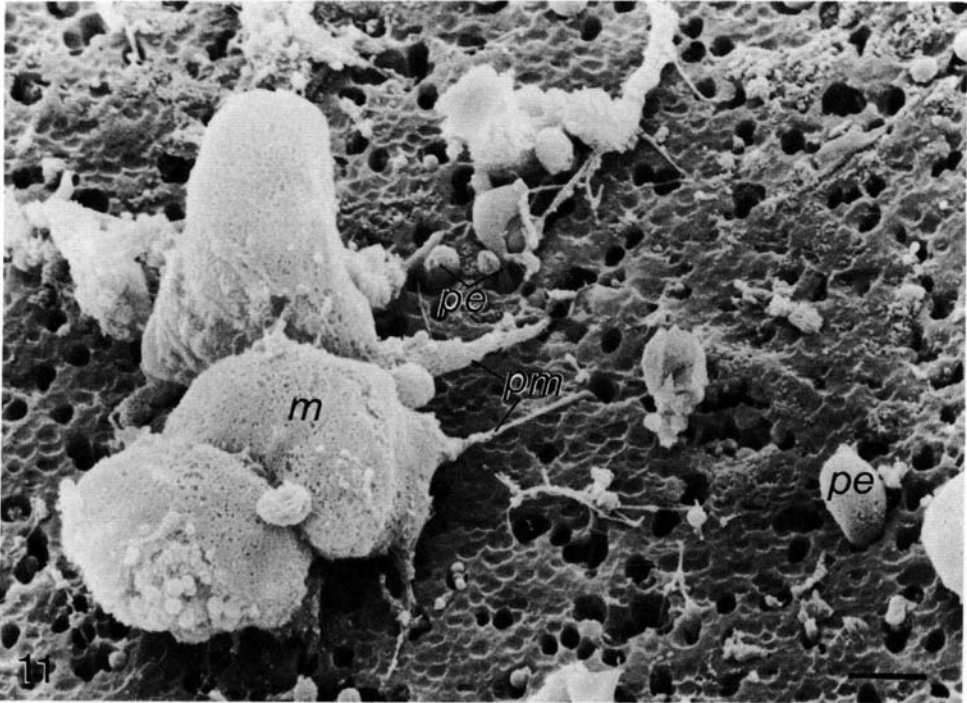


Fig. 11. A scanning electron micrograph of the surface of a  $0.8\ \mu\text{m}$  porosity Nuclepore filter on which mesenchyme had been cultured transfilter to epithelium. Processes (pm) from the mesenchymal cells (m) can be seen entering the pores. Processes from the underlying epithelium (pe) have emerged and lie close to the mesenchymal cells. Bar =  $2\ \mu\text{m}$ .

of filters of  $0.8$ ,  $0.4$  and  $0.1\ \mu\text{m}$  porosity (Fig. 11) but not penetrating those of  $0.03\ \mu\text{m}$  porosity. As is indicated in Fig. 11, epithelial cell processes completely traversed the  $10\ \mu\text{m}$  thick filters to lie close to mesenchymal cell processes. No evidence of the deposition of extracellular matrix products was seen either on the epithelial or on the mesenchymal sides of these filters (Figs 8–11).

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Fig. 8. A scanning electron micrograph of the underside of a  $0.8\ \mu\text{m}$  Nuclepore filter showing epithelial cell processes (cp) penetrating the pores (p) of the filter after 24 h in culture. Bar =  $1\ \mu\text{m}$ .

Fig. 9. Scanning electron micrograph of the underside of a  $0.4\ \mu\text{m}$  Nuclepore filter after 24 h in culture. The epithelial cell processes (cp) are smaller and fewer than those shown in Fig. 8 but the area over which they were observed was about the same. Bar =  $0.5\ \mu\text{m}$ .

Fig. 10. A scanning electron micrograph of the underside of a  $0.1\ \mu\text{m}$  Nuclepore filter which was cultured for 24 h. A few very slender epithelial cell processes (cp) were observed on this filter. These processes were about the same diameter as the pores through which they passed. The processes were sparsely distributed in a small area of this filter. Epithelial cell processes were not observed penetrating the other two  $0.1\ \mu\text{m}$  Nuclepore filters examined. Bar =  $0.5\ \mu\text{m}$ .

## DISCUSSION

Control experiments verified previous studies (Tyler & Hall, 1977) demonstrating that the H. H. stage-22 mandibular mesenchyme requires an epithelial induction in order to form bone and that the experimental procedures employed to separate and recombine these tissues did not inhibit the induction.

The interpretation of results from transfilter experiments requires the following consideration: there is a lag *in vitro* of 7 to 9 days between the time the induction actually takes place and the time the results of that induction, osteogenesis, can be first detected histologically (Hall, 1978a; Tyler & Hall, 1977). When transfilter preparations were set up as shown in Fig. 1, the epithelium lay flat against the filter, directly beneath the isolated mesenchyme which had been placed on the opposite side of the filter. During the development of the transfilter method employed in the present study, explants were sectioned and examined after different periods in culture. The epithelium remained in its original position for 24 to 48 h (Van Exan & Hall, unpublished data), a period sufficient for the induction to take place. By the time the response to that induction was detectable (day 7 in CAM grafts and day 9 in cultures) the epithelium had undergone differentiation and curled into a ball, frequently sinking into the mesenchyme of the intact mandible and losing its direct contact with the filter.

A second consideration is any possible effect of the mesenchyme of the intact mandible included as a control with the transfilter recombinations. Again, in developing the procedure used in the present study, experiments were conducted to show that mandibular mesenchyme which had received an epithelial induction (from embryos of H. H. stage 24) could not induce bone formation when cultured transfilter to a second piece of mesenchyme which has not received an epithelial induction. Thus the presence of the mesenchyme in the intact, control mandible, could not account for the induction of isolated mesenchyme. Any induction which occurred must have been from the epithelium. The intact mandible did however provide a necessary substrate for the healthy maintenance of the isolated epithelium. Mandibular mesenchyme provides the epithelium with a factor or factors required to maintain the epithelium in an unkeratinized state (Tyler & Hall, 1977). With these considerations in mind, the effects of the interposing filters in blocking or permitting epithelial induction of bone in the mandibular mesenchyme were examined. Induction of bone was not observed when mandibular mesenchyme was separated from epithelium by 0.45  $\mu\text{m}$  porosity, 25 or 150  $\mu\text{m}$  thick Millipore filters. Failure of osteogenesis was attributed to inability of mesenchymal and epithelial cell processes to approach one another through the tortuous channels which meander through Millipore filters. This is in contrast to the induction of cartilage and bone in postfoetal mammals where bone matrix-derived protein can act both across filters (150–750  $\mu\text{m}$  thickness, 0.45  $\mu\text{m}$  porosity) and by diffusion through culture

medium (Nogami & Urist, 1975; Nakagawa & Urist, 1977; Urist *et al.* 1977).

Epithelial induction of mandibular bone was blocked in the present study when a nuclepore filter of 0.03  $\mu\text{m}$  pore diameter was placed between the epithelium and its mesenchyme. Scanning electron microscopy demonstrated that the 0.03  $\mu\text{m}$  pores were small enough to block the penetration of epithelial cell processes. Pores of this size were large enough to permit the passage of a diffusible cell product and these filters are only 5  $\mu\text{m}$  thick. Failure of an induction response in these transfilter explants further supports the contention that the mechanism of induction does not involve a diffusible substance. If it does, diffusion must be over distances smaller than 5  $\mu\text{m}$ .

A distinct correlation was observed between the ability of epithelial cell processes to penetrate the Nuclepore filter and the ability of the epithelium to transmit an inductive message through the filter. A similar correlation has been demonstrated in transfilter studies of eye (Meier & Hay, 1975; Hay & Meier, 1976; Hay, 1977) odontoblast (Thesleff, 1977), kidney tubule (Wartiovaara *et al.* 1974) and chick limb bud and scleral cartilage differentiation (Gumpel-Pinot, 1980; Smith & Thorogood, 1983). These observations demonstrate the need for direct tissue apposition in a number of diverse epithelial–mesenchymal interactions. This implies that the site of inductive activity is restricted to the epithelial–mesenchymal interface and that the mechanism of induction may act only over a relatively short distance. It has been postulated that such an inductive mechanism may involve direct cell–cell contact or cell–matrix interactions (Hay & Meier, 1976; Gumpel-Pinot, 1980; Saxen, 1977).

Although we conducted an exhaustive ultrastructural study of the epithelial–mesenchymal interface in the chick mandible, we were unable to detect any evidence of direct contact between cell membranes of epithelial and mesenchymal cells during inductive or non-inductive stages of development *in ovo* (Van Exan & Hall, 1983). We did, however, observe that the mesenchyme was separated from its epithelium by a continuous basal lamina and that the mesenchymal cells made numerous contacts with the basal lamina usually through long slender cell processes. Similar observations have been reported in the developing chick limb bud. The limb-bud epithelium is separated from its mesenchyme by a continuous basal lamina between H. H. stages 10 and 26 (Jurand, 1965; Berczy, 1966; Ede *et al.* 1974; Smith *et al.* 1975; Kaprio, 1977). Limb-bud cartilage fails to differentiate without an epithelial induction – an induction which only occurs when the two tissues are in direct contact with each other (Gumpel-Pinot, 1980). These observations suggest an inductive mechanism other than direct epithelial–mesenchymal cell contact and favour a cell–matrix mechanism with possible involvement of the basal lamina.

Our first study in this series further substantiates this hypothesis (Hall & Van Exan, 1982). When isolated mesenchyme was cultured on Millipore filters on which epithelial extracellular cell products had been previously deposited, the mesenchyme responded as if it had been placed on living epithelial cells by

forming bone. These experiments provided direct evidence that epithelial cell products deposited on a Millipore filter are inductively active. Indirect evidence that the inductive activity of the epithelium resides in its basal lamina was provided by the fact that the cell products bore a striking resemblance to basal laminae when viewed ultrastructurally. Treatment of the cultured epithelial cells with trypsin or LACA both inhibited the inductive activity of the epithelial cell products and removed the basal lamina-like substance from the filters. Trypsin non-selectively degrades protein while LACA specifically inhibits the hydroxylation of proline, resulting in the formation of under-hydroxylated collagen whose release from the cell is impaired (Takeuchi & Prockop, 1968). Previous experiments also implicated collagen as a possible active component of the inductive system (Bradamante & Hall, 1980).

Basal laminae have been implicated in the epithelial-mesenchymal interactions involved in the initiation of several other tissues and organs, including scleral cartilage (Newsome, 1976), limb-bud cartilage (Gumpel-Pinot, 1980), somatic cartilage (Hall, 1977) and teeth (Lesot *et al.* 1981; Thesloff & Hurmerinta, 1981). The only one of these tissues which has been experimentally induced in response to extracellular products is scleral cartilage (Newsome, 1976). In our most recent experiments (Hall *et al.* 1983), more direct evidence has been found to support the role of basal lamina in epithelial induction of osteogenesis in the chick mandible. Mandibles removed from stage-22 chick embryos were treated with EDTA. The epithelium was removed but the basal lamina remained intact on the mandibular mesenchyme. Thirty percent of explants treated in this way formed bone when CAM-grafted for 8 days. These results indicate that the basal lamina or some component(s) thereof may contain the inductive message which is subsequently recognized by the mesenchyme cells. Confirmation of this hypothesis awaits the results of continuing studies aimed at isolating specific components of the basal lamina which exhibit inductive activity.

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