Both Humoral Mesenchymal Factors and the Close Association between the Hepatic Endoderm and Mesenchyme can be Involved in Liver Formation of Mouse Embryos

Authors: Abe, Masayoshi, and Shiojiri, Nobuyoshi

Source: Zoological Science, 17(5) : 633-641

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.633

ZOOLOGICAL SCIENCE **17**: 633–641 (2000)

Both Humoral Mesenchymal Factors and the Close Association between the Hepatic Endoderm and Mesenchyme can be Involved in Liver Formation of Mouse Embryos

Masayoshi Abe and Nobuyoshi Shiojiri*

Department of Biology, Faculty of Science, Shizuoka University, Oya 836, Shizuoka 422-8529, Japan

ABSTRACT—Previous studies with tissue recombination experiments demonstrated that the splanchnic mesenchymes, including hepatic, pulmonary and stomach mesenchymes can support hepatocyte differentiation from the hepatic endoderm in 9.5-day mouse embryos. This phenomenon corresponds to the second hepatic induction. The present study was undertaken to determine whether direct cell-cell contacts between the hepatic endoderm and mesenchyme are required for hepatocyte differentiation, using transfilter experiments in which membrane filters with various pore sizes were inserted between the endoderm and the hepatocyte-inducing mesenchyme (the chick lung mesenchyme). Hepatocyte differentiation occurred even when the direct cell-cell contacts between the hepatic endoderm and the mesenchyme were absent, suggesting that humoral factors may work in this interaction. However, growth of hepatocytes was most prominent in the transfilter experiments with filters having pore sizes of 0.2 and 0.8 μm, which permitted mesenchymal cells or their cell processes to penetrate to the side of the endoderm. These results suggest that two types of tissue interactions, including humoral mesenchymal factors and very local tissue interactions such as direct cell-cell contacts, may be involved in the second step of hepatic induction.

INTRODUCTION

Liver formation from the endoderm requires two successive inductive events with the cardiac mesoderm and the septum transversum mesenchyme (hepatic mesenchyme) during avian and mammalian development (Le Douarin, 1975; Houssaint, 1980; Fukuda-Taira, 1981; Gualdi *et al*., 1996; Koike and Shiojiri, 1996). Recent gene-knockout studies suggested another step of the endodermal-mesodermal interactions in liver formation, which follows the preceding inductions and includes the involvement of transcription factor *Hlx* expressed in mesenchymal tissue (Hentsch *et al*., 1996). Several liver-enriched transcription factors, including HNF-6 and HNF-4, are expressed in endodermal cells during liver primordium formation (Duncan *et al*., 1994; Cereghini, 1996; Landry *et al*., 1997; Rausa *et al*., 1997). However, it still remains to be revealed not only what kinds of signaling molecules work between the endoderm and the mesoderm, but also what endodermal-mesodermal interactions (i.e. humoral or direct cell-cell contacts) occur in these inductive phenomena.

During development of several organs, including the salivary gland, lung, kidney, and stomach, diffusible growth factors from the mesenchyme stimulate growth, morphogenesis and cell differentiation of their epithelial cells (Fukamachi *et al*., 1994; Nogawa and Ito, 1995; Post *et al*., 1996; Vukicevic *et al*., 1996; Koike and Yasugi, 1999). Direct cell-cell contacts and extracellular matrices have also been shown to play crucial roles in these organogenesis systems (Schuger *et al*., 1996; Kadoya *et al*., 1997; Müller *et al*., 1997; Yang *et al*., 1998). To clarify the molecular mechanisms of hepatic induction, it is important to know whether humoral factors or direct cell-cell contacts are required for the tissue interactions.

To determine whether direct cell-cell contacts are essential for inductive events, transfilter experiments in which the inducer and the responding tissue are allocated on both sides of membrane filters with various pore sizes are useful (Takiguchi-Hayashi and Yasugi, 1990; Takahashi and Nogawa, 1991). Porous membrane filters, which can regulate migration of cells and their cell processes across them, are commercially available.

In the present study, we performed transfilter experiments to examine the roles of humoral factors and direct cell-cell contacts in the second step of the hepatic induction of the mouse embryo. We used the chick lung mesenchyme as the inducer in place of the mouse hepatic mesenchyme in the transfilter experiments, because it possesses potency to support hepatocyte differentiation from the hepatic endoderm in vitro (Koike and Shiojiri, 1996), and it can easily be isolated as compared with the tiny hepatic mesenchyme. The present transfilter experiments demonstrated that humoral mesenchymal factors alone could induce hepatocyte differentiation from the hepatic endoderm, but the growth of hepatocytes in this

* Corresponding author: Tel. +81-54-238-4780;
FAX. +81-54-238-0986.
E-mail sbnshio@ipc.shizuoka.ac.jp

case was very poor as compared with the culture of the hepatic endoderm that could directly contact the mesenchymal cells.

MATERIALS AND METHODS

Animals

C3H/HeSlc strain mice (SLC, Shizuoka, Japan) were used. Animals were mated during the night, and pregnancy was confirmed by the presence of a vaginal plug the next morning. Noon of the day the vaginal plug was found was considered 0.5 days of gestation. Embryos at 9.5 days of gestation were staged according to their somite number. Fertilized chick eggs were obtained from a local breeder (Yamagishi, Tsu, Japan).

Transfilter experiments

Mouse embryos at 9.5 days of gestation (with 15-23 somites) were dissected in HEPES (Nakarai Chemical Co. Ltd., Tokyo, Japan)-buffered Dulbecco's modified MEM (DMEM; Gibco, Grand Island, N. Y., USA) under a dissection microscope (model SZH131; Olympus, Tokyo, Japan) with illuminators (model LGW; Olympus), and liver primordia were isolated.

The endoderm of the liver primordium was separated from the hepatic mesenchyme using 0.01% collagenase (for cell preparation; Wako Pure Chem. Ind., Tokyo, Japan) (Fig. 1). Lung mesenchyme was prepared from 4-day chick embryos also with the aid of collagenase. The hepatic endoderm, which was covered with Matrigel (Collaborative Research, Bedford, Mass., USA), was placed on one side of membrane filters with various pore sizes (Nuclepore, Pleasanton, CA, USA), and the chick lung mesenchyme was placed on the other side (Fig. 1B). Membrane filters with three pore sizes (0.05 μm, 0.2 μm and 0.8 μm; 10 μm in thickness) were used in the transfilter experiments. For the membrane filters with the pore size of 0.05 μm only diffusible molecules, not cell processes or cell bodies, could pass through. The tissue recombinants for the transfilter experiments were placed on membrane filters (RA-type; Nihon Millipore Kogyo K. K., Yonezawa, Japan) on stainless steel grids, and were cultured for 5 days at the interface between the culture medium and the gas phase of 5% CO_2 in air (Fig. 1A)(Koike and Shiojiri 1996). DMEM supplemented with 10% fetal bovine serum (Gibco), epidermal growth factor (10 ng/ml; Upstate Biotechnology Inc., Lake Placid, N.Y., USA), insulin (5 μg/ml; Sigma, St Louis, Mo., USA), dexamethasone (10^{-7}M; Sigma), penicillin (100 U/ml; Meiji Seika Co. Ltd., Tokyo, Japan), and streptomycin (100 μg/ml; Meiji Seika Co. Ltd.) was used as the culture medium. The hepatic endoderm, the chick lung mesenchyme, the direct recombinants of the hepatic endoderm and lung mesenchyme, and the liver primordium were also cultured in vitro as controls (Fig. 1).

To assess migration of mesenchymal cells across the membrane filter in the transfilter experiments, lung mesenchymes were cultured alone on the Nuclepore filter without the endoderm for 3, 6, 12 and 24 hr (Fig. 1D), and the surface ultrastructure on the other side of the membrane filter was observed by scanning electron microscopy.

Histochemistry

Expression of alpha-fetoprotein (AFP), albumin and carbamoylphosphate synthetase I (CPSI), and glycogen accumulation was his-

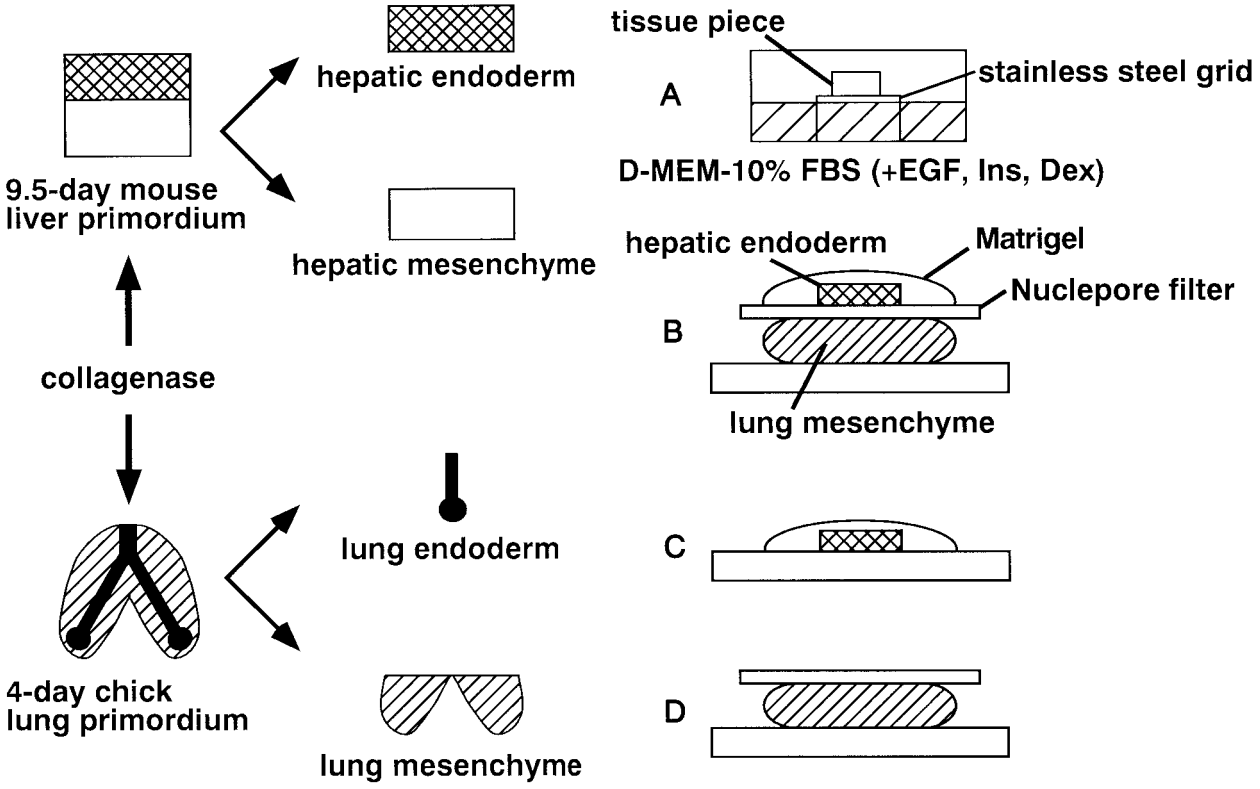

Fig. 1. Schematic design of the transfilter experiments. The hepatic endoderm and lung mesenchyme were isolated from 9.5-day mouse liver primordium and 4-day lung primordium, respectively, with the aid of collagenase. The hepatic endoderm was placed on one side of membrane filters with one of three pore sizes (0.05, 0.2 and 0.8 μm; 10 μm in thickness), and the lung mesenchyme was placed on the other side (B). The hepatic endoderm (C), the lung mesenchyme (D), the direct recombinants of the hepatic endoderm and the lung mesenchyme, and the liver primordia were cultured as controls. The direct recombinants of the hepatic endoderm and the lung mesenchyme, and the liver primordia are not shown in this figure. These tissue pieces were cultured for 5 days in the interface of the medium and the gas phase (A).

tochemically examined in the explants as hepatocyte differentiation markers (Koike and Shiojiri, 1996). Tissues were fixed in a cold mixture of ethanol and glacial acetic acid (99:1 v/v) overnight, and embedded in paraffin at 53°C. Paraffin sections were cut at 6 μm thickness. Expression of AFP, albumin and CPSI was examined immunohistochemically. Dewaxed sections were incubated with the normal goat serum (Rockland, Gilbersville, PA, USA)(1/20 dilution with phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA]) for 20 min, and then with rabbit anti-mouse AFP antiserum (ICN Biomedicals, Inc., Aurora, OH, USA) (1/50 dilution in PBS-1% BSA), anti-mouse albumin antiserum (Cappel Research Products, Durham, NC, USA) (1/50 dilution), or anti-rat CPSI antiserum (a generous gift from Dr. W. H. Lamers)(Gaasbeek Janzen *et al*., 1985) (1/1000 dilution) for 1 hr at room temperature. After being thoroughly washed in PBS, sections were then incubated with FITC-labeled goat anti-rabbit IgG antibodies (Cappel Research Products)(1/100 dilution in PBS-1% BSA) for 1 hr at room temperature. Specific immunofluorescence was observed with a fluorescent microscope (Model BHS-RF; Olympus). As negative controls, sections were incubated with the normal rabbit serum diluted with PBS-1% BSA in place of the primary antibody.

For demonstration of glycogen, periodic acid-Schiff (PAS) staining was carried out according to the method of McManus (1948).

Quantitation of growth of hepatocytes

Areas of PAS-positive hepatocytes in every fifteen sections of the explants were traced onto transparent sheets, input and then quantitated with a Luzex F image analysis system (Nireco Corporation, Hachioji, Japan). The data were expressed as volumes which hepatocytes occupied in explants, and statistically analyzed by t-test. Differences were considered significant when P was smaller than 0.05.

Scanning electron microscopy

Tissues were fixed with membrane filters in 2% glutaraldehyde-2.25% paraformaldehyde buffered with 0.01M phosphate buffer (pH 7.2) overnight at 5°C, dehydrated with alcohol series and immersed in t-butyl alcohol. Dehydrated tissues were dried with a freeze-drier (ES-2030; Hitachi, Tokyo, Japan) after being frozen in t-butyl alcohol at –20°C, and then coated with gold by an ion coater (E-1020; Hitachi). The surface ultrastructure of the membrane filter on the other side of the mesenchyme, which was placed on one side of it at the start of the culture (Fig. 1D), was observed by scanning electron microscopy (S-3200; Hitachi).

RESULTS

1. Analysis of hepatocyte differentiation by transfilter experiments

When the liver primordium was cultured in vitro for 5 days, the endodermal cells differentiated into many large hepato-

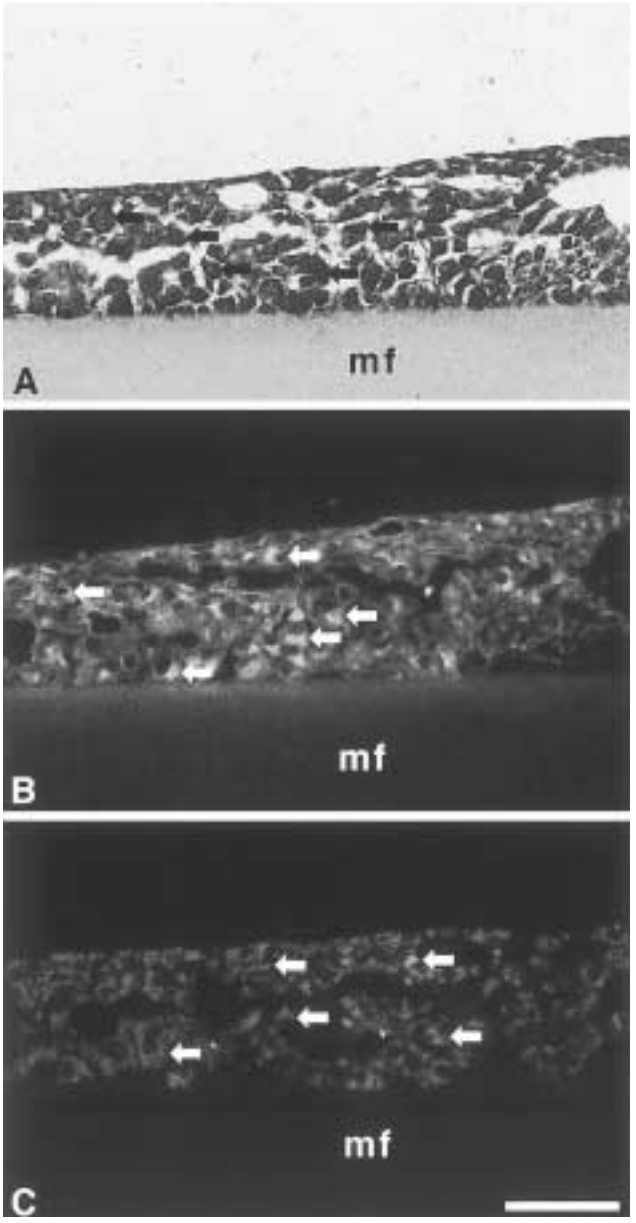

Fig. 2. Hepatocyte differentiation in 9.5-day liver primordia cultured in vitro for 5 days. (A) PAS-hematoxylin (HX) staining. (B) Albumin immunostaining. (C) CPSI immunostaining. The endodermal cells differentiate into hepatocytes (arrows) expressing albumin and CPSI, and accumulating glycogen. mf, Millipore filter. Bar indicates 50 μm.

Table 1. Differentiation of hepatocytes in the transfilter experiments.

Transfilter experiments				Culture of endoderm without mesenchyme
Pore size (μm)	0.05	0.2	0.8	
No. of experiments	16	8	15	9
No. of explants in which endodermal cells were alive	9	7	15	0
No. of explants in which hepatocytes differentiated	6	4	9	0
No. of explants in which epithelial structures with large lumina were formed	2	5	11	0
No. of explants showing migration of mesenchymal cells to the opposite side*	0	0	15	*

* Migration of mesenchymal cells to the opposite side in the transfilter experiments at the light microscopic level.
* Mesenchymal cells were not co-cultured with the hepatic endoderm.

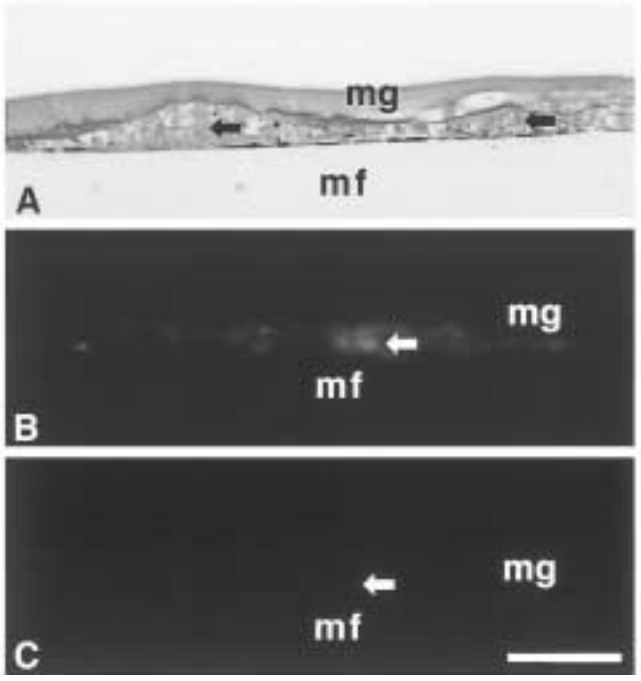

Fig. 3. Culture of the hepatic endoderm alone for 5 days. (A) PAS-HX staining. (B) AFP immunostaining. (C) Albumin immunostaining. The hepatic endodermal cells are dead in the absence of the mesenchyme. They are negative for albumin immunostaining and PAS staining. Arrows indicate hepatic endodermal cells. mf, Millipore filter; mg, Matrigel. Bar indicates 50 μm.

cytes expressing AFP, albumin and CPSI, and accumulating glycogen heavily (Fig. 2). They also formed epithelial structures with large lumina lined by the connective tissue, which morphologically resembled the immature stomach and never expressed albumin and CPSI. When the hepatic endoderm was cultured in the absence of mesenchyme for 5 days, the cells were mostly round and dead (Table 1, Fig. 3). The hepatic endodermal cells were positively stained for AFP, but never expressed albumin or CPSI. The PAS staining for glycogen was negative in these endodermal cells. In contrast, when the isolated hepatic endoderm was directly recombined with the chick lung mesenchyme and cultured, it differentiated into many hepatocytes expressing AFP, albumin and CPSI, and accumulating glycogen, and also into epithelial cells surrounding large lumina.

When a membrane filter with a pore size of 0.8 μm was inserted between the hepatic endoderm and the lung mesenchyme, and the recombinants were cultured in vitro for 5 days, the endodermal cells formed large luminal structures and also differentiated into many large hepatocytes expressing AFP, albumin and CPSI, and accumulating glycogen (Tables 1 and 2, Fig. 4). However, not all explants exhibited hepatocyte differentiation in these transfilter experiments. Epithelial cells forming large luminal structures were columnar or cuboidal, and were negative for albumin and CPSI immunostaining. Lung mesenchymal cells migrated across the porous membrane, and were closely associated with the epithelial structures, and also with differentiated hepatocytes that were located near the membrane filter. However, many hepatocytes that were located in other areas of the explants did not contact these mesenchymal cells. The endodermal cells did not migrate to the side of the mesenchyme across the membrane filter.

In transfilter experiments using membrane filters with the pore size of 0.2 μm, through which the cell processes of mesenchymal cells could penetrate, many large hepatocytes also differentiated (Tables 1 and 2, Fig. 4). The growth and differentiation patterns of hepatocytes in these experiments were similar to those in the culture with filters with the pore size of 0.8 μm, though the size of the differentiated epithelial structures was smaller than that in the 0.8 μm-filter experiment. At the light microscopic level, migration of mesenchymal cells across the membrane filter could not be confirmed.

In contrast, when membrane filters with a pore size of 0.05 μm were used in the transfilter experiments, large hepatocytes could differentiate (Table 1, Fig. 5), but their growth was very poor as compared with the cultures with filters having pore sizes of 0.8 and 0.2 μm (Table 2). The differentiation state of the hepatocytes was a little immature or similar as

Table 2. Histochemical analysis of the differentiation state and growth of hepatocytes in the transfilter experiments. [¶]

	Transfilter experiments			Culture of endoderm without mesenchyme
Pore size (μm)	0.05	0.2	0.8	
AFP	ND	ND	ND	++ (1/1)[a]
Albumin	+〜++ (6/6)	++ (4/4)	++ (8/8)	– (0/9)
CPSI	+〜++ (4/6)	++ (4/4)	++ (7/8)	– (0/9)
glycogen	+〜++ (4/6)	++ (4/4)	++ (7/8)	– (0/9)
hepatocyte population size ($\times 10^7$ μm^3)[b]	0.11 ± 0.05[c*] (6)[d]	1.43 ± 0.53* (4)	1.64 ± 0.96 (4)	

[¶] Explants of transfilter experiments in which hepatocytes morphologically differentiated (see Table 1) were histochemically analyzed.

–, no staining; +, moderate staining; ++, strong staining. ND, not determined.

[a] Number of explants in which positive hepatocytes differentiated among explants examined.

[b] Hepatocyte population sizes were expressed as volumes which PAS-positive large hepatocytes occupied in explants.

[c] Data are expressed as means±SE. The means indicated by asterisks are statistically significant ($P<0.05$).

[d] The numbers in parenthesis show the numbers of explants examined.

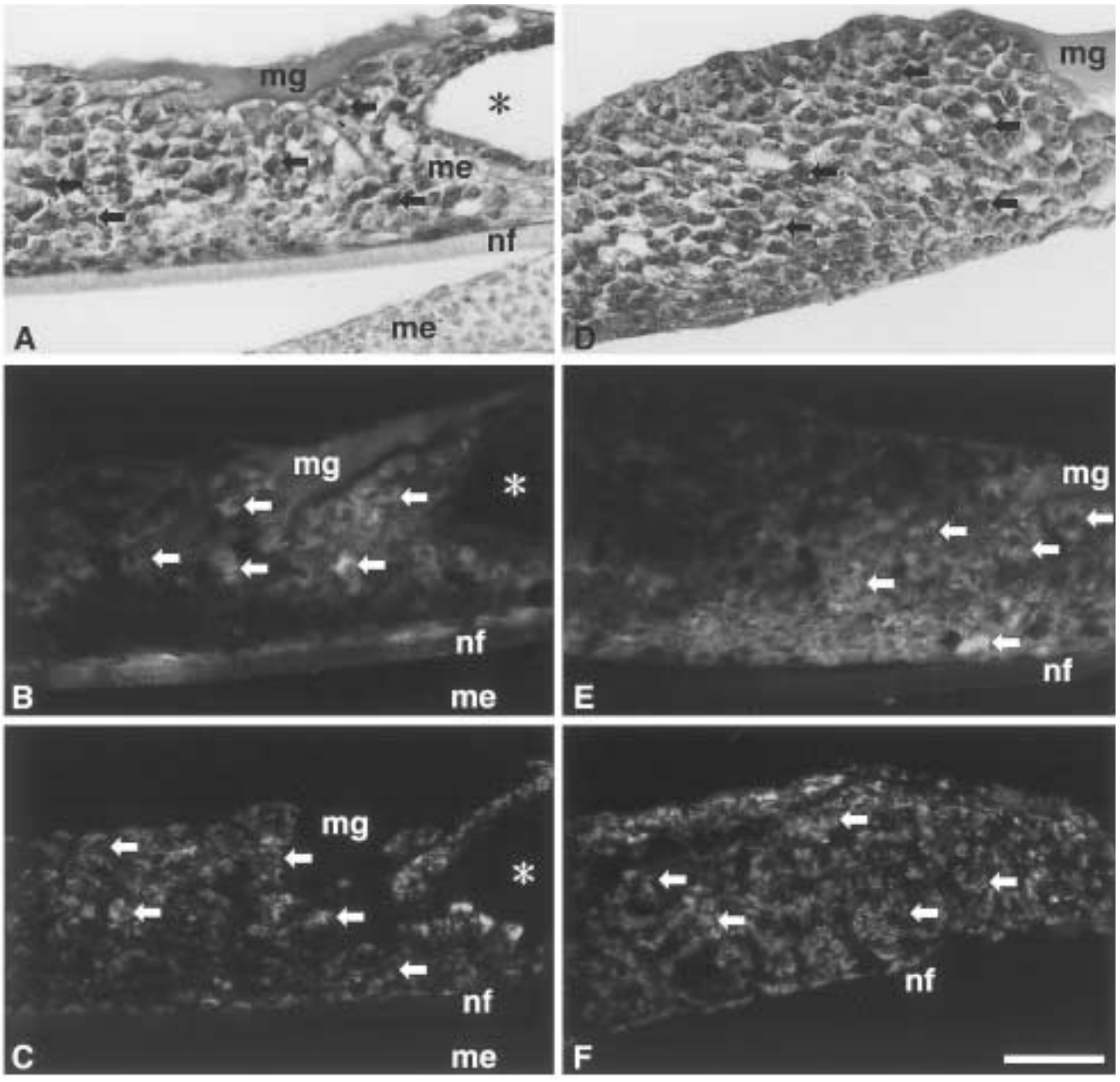

Fig. 4. Transfilter experiments with membrane filters having 0.8 μm pores (A, B, C) and 0.2 μm pores (D, E, F). (A, D) PAS-HX staining. (B, E) Albumin immunostaining. (C, F) CPSI immunostaining. The hepatic endodermal cells differentiate into many large hepatocytes (arrows) expressing albumin and CPSI, and accumulating glycogen in both transfilter experiments with the membrane filters having 0.8 μm pores and 0.2 μm pores. They also formed a large luminal structure (asterisks in A, B, and C). Although the lung mesenchymal cells that migrated across the membrane filter are adjacent to the large luminal structures, they do not invade the area of hepatocytes (A). All cells seen in D are PAS-positive hepatocytes. Mesenchymal cells are also abundant under the Nuclepore filter (A). In the transfilter experiment with a membrane filter having 0.2 μm pores, the lung mesenchyme was accidentally removed in the process for histochemistry. me, mesenchymal cells; mf, Millipore filter; mg, Matrigel; nf, Nuclepore filter. Bar indicates 50 μm.

compared with that of the cultures with filters with pore sizes of 0.8 and 0.2 μm (Table 2). The large epithelial structures found in the transfilter experiments with larger pore sizes were rarely observed, though small ones sometimes developed in this culture. Migration of mesenchymal cells to the side of the endoderm did not occur.

Tables 1 and 2 summarize the growth and differentiation of hepatocytes in these transfilter experiments. More hepatocytes differentiated with the increase of the pore size of the membrane filters in the explants for the transfilter experiments. The number of hepatocytes that differentiated in the transfilter experiments with 0.8 and 0.2 μm was roughly 13 times as large as that seen in the experiments with 0.05 μm filters (Table 2).

The growth and organization of lung mesenchymal cells were also dependent on the pore size of the membrane filter

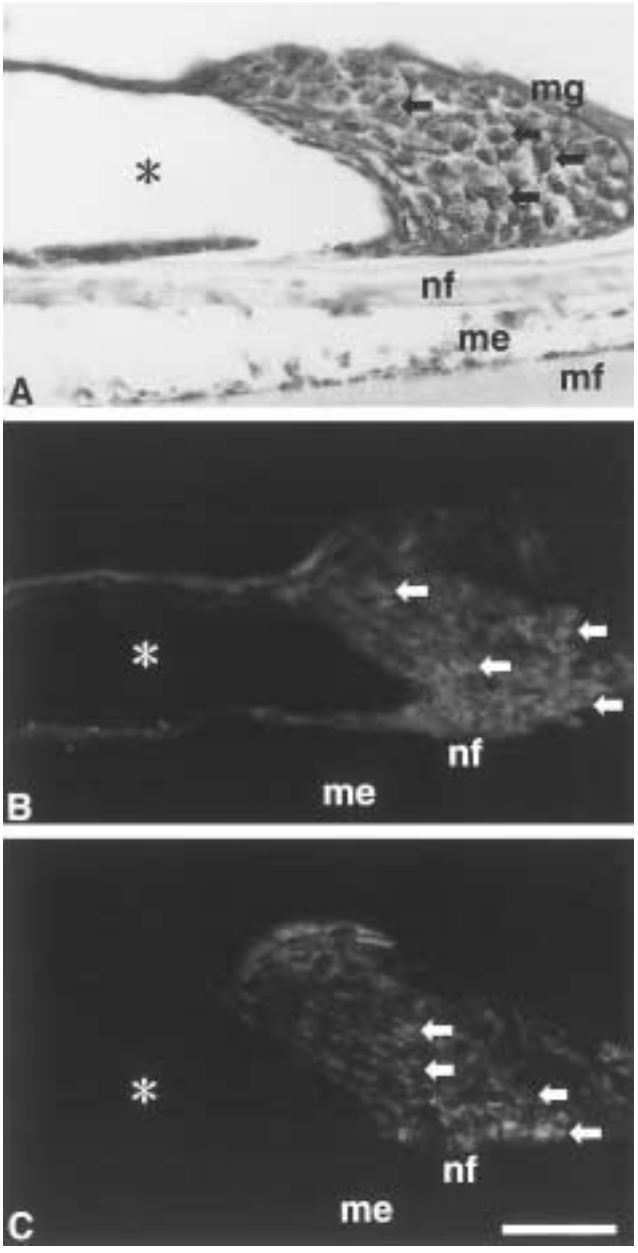

Fig. 5. Transfilter experiment with a membrane filter having 0.05 μm pores. (A) PAS-HX staining. (B) Albumin immunostaining. (C) CPSI immunostaining. The hepatic endodermal cells differentiate into large hepatocytes (arrows) expressing albumin and CPSI, and accumulating glycogen. The number of large hepatocytes is poor as compared with those of the culture with the membrane filters having 0.8 and 0.2 μm pores (Fig. 4). Also note the poor development of the lung mesenchymal cells (me) in this culture. Asterisks indicate a luminal structure. mf, Millipore filter; mg, Matrigel; nf, Nuclepore filter. Bar indicates 50 μm.

in these transfilter experiments. The smaller pore sizes gave poor growth and low cell density of the mesenchymal cells (Fig. 4A, 5A). The mesenchymal cells also had a tendency to concentrate near the epithelial structures forming large lumina. When the lung mesenchyme was cultured alone for 5 days in vitro, most cells could not survive.

2. Migration of lung mensenchymal cells across the porous membrane filter

Although the lung mesenchymal cells could be observed to migrate across the membrane filter with the pore size of 0.8 μm in the transfilter experiments at the light microscopic level, the migration of the endodermal cells to the side of the mesenchyme did not occur. Thus, we examined which membrane filters the lung mesenchymal cells could migrate across, and how fast the migration occurred when they were cultured alone on one side of the membrane filter.

Scanning electron microscopy revealed that extensive migration of mesenchymal cells to the other side of the membrane filters took place when the pore size was 0.8 μm (Fig. 6A, 6B). The cell processes of mesenchymal cells penetrated through the membrane filters with a pore size of 0.2 μm (Fig. 6C, 6D) but not the cell bodies. The speed of the migration and penetration was very rapid and occurred within 3 hr of culture. Deposition of extracellular matrices by the mesenchymal cells was not clear in these cultures. However, neither mesenchymal cells themselves nor their cell processes migrated across the membrane filter with 0.05 μm pores (Fig. 6E). Our scanning electron microscopical technique could not visualize the 0.05-μm pores due to the coating.

DISCUSSION

During tissue interactions in organogenesis of mammalian and avian embryos, humoral factors and very local interactions, including direct cell-cell contacts, can play important roles (Nogawa and Ito, 1995; Post *et al*., 1996; Schuger *et al*., 1996; Kadoya *et al*., 1997; Müller *et al*., 1997; Yang *et al*., 1998). In liver formation, two or three successive endodermal-mesodermal interactions are crucial for development (Le Douarin, 1975; Houssaint, 1980; Fukuda-Taira, 1981; Gualdi *et al*., 1996; Hentsch *et al*., 1996; Koike and Shiojiri, 1996). In the present study, we could divide tissue interactions of the second hepatic induction into two types; one with humoral factors, and the other with very local interactions, including direct cell-cell contacts. We demonstrated that humoral factors from the mesenchyme were effective for survival and differentiation of hepatic endodermal cells, but their growth was poor in the transfilter experiments using the Nuclepore filters that did not permit mesenchymal cells or their cell processes to penetrate their pores. The differentiation state of hepatocytes in the culture with such membrane filters was a little immature or similar as compared with the cultures in which direct cell-cell contacts between the hepatic endodermal cells and lung mesenchymal cells were permitted. It remains to be clarified whether the humoral factors work as survival factors or differentiation factors, or both, because in the absence of mesenchyme the endodermal cells failed to survive in vitro. Prominent growth of hepatocytes was induced in the transfilter experiments in which direct cell-cell contacts were permitted, though not all explants exhibited hepatocyte differentiation in these cultures.

It is well known that diffusible growth factors mediate tis-

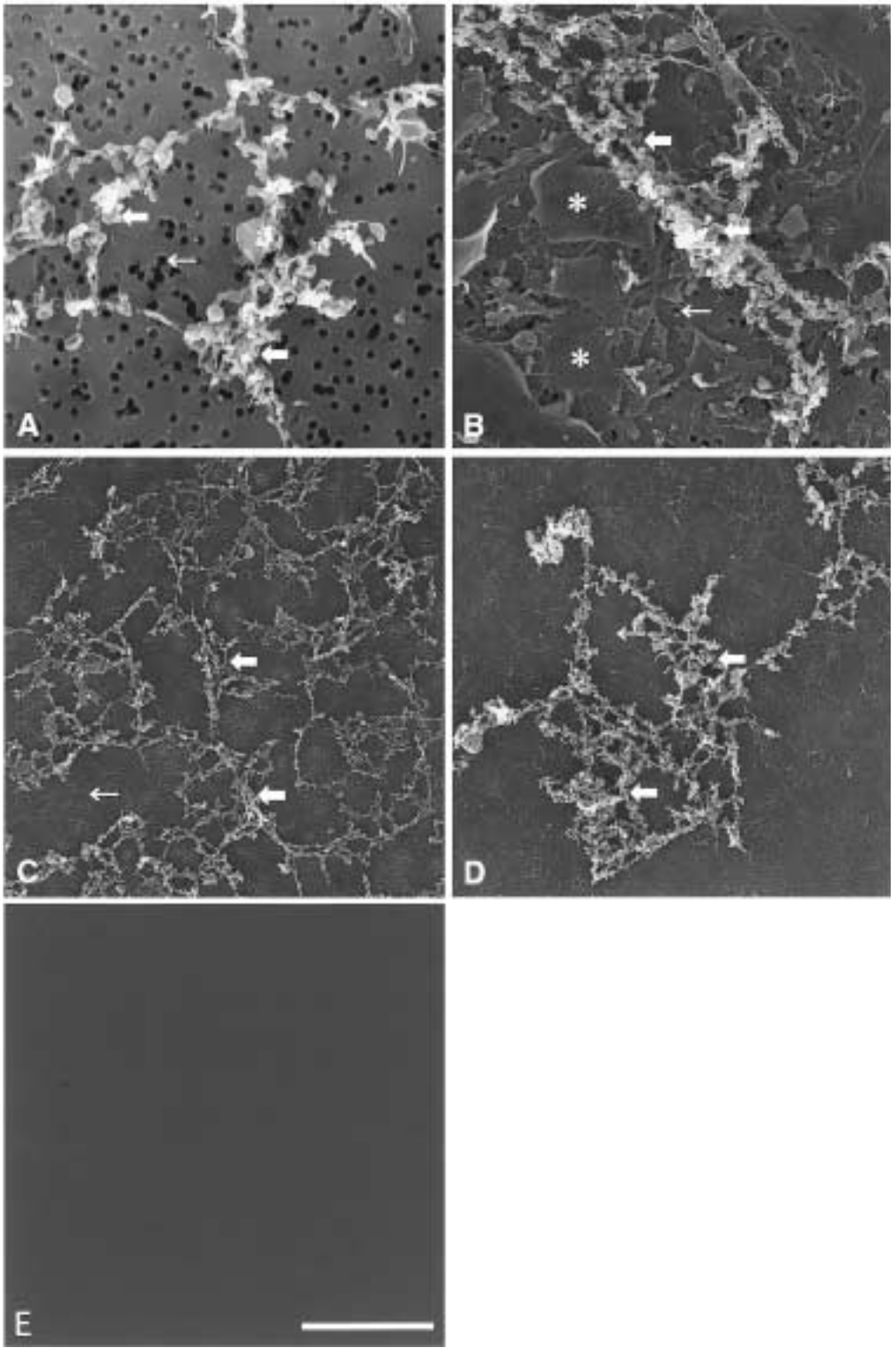

Fig. 6. Migration of lung mesenchymal cells and penetration of their cell processes through the membrane filters used in the transfilter experiments. Lung mesenchymal cells were cultured alone on one side of the membrane filters, and the surface ultrastructure on the other side of the membrane filter was observed with scanning electron microscopy. (A) After 3 hr of culture with the membrane filter having 0.8 μm pores, the lung mesenchymal cell processes penetrate (thick arrows) to the opposite side. (B) After 12 hr of culture with the membrane filter having 0.8 μm pores, migrated lung mesenchymal cells (asterisks) cover the opposite side of the membrane filter. (C) After 3 hr of culture with the membrane filter with 0.2 μm pores, the lung mesenchymal cell processes also penetrate (thick arrows) to the opposite side. (D) After 12 hr of culture with the membrane filter with 0.2 μm pores, cell processes (thick arrows) are observed on the opposite side, but cell bodies are not seen. (E) After 3 hr of culture with the membrane filter with 0.05 μm pores, the lung mesenchymal cell processes cannot penetrate through the pores. The 0.05 μm pores cannot be observed. Small arrows indicate pores of the membrane filters. Bar indicates 5 μm.

sue interactions in several systems of organogenesis (Fukamachi *et al*., 1994; Nogawa and Ito, 1995; Post *et al*., 1996; Vukicevic *et al*., 1996; Koike and Yasugi, 1999). Hepatic mesenchyme, lung mesenchyme and stomach mesenchyme can induce hepatocyte differentiation in hepatic endodermal cells at the second step of the hepatic induction, but somitic mesenchyme and metanephric mesenchyme are impotent (Le Douarin, 1975; Fukuda-Taira, 1981; Koike and Shiojiri, 1996). Although diffusible growth factors produced in the hepatocyte-inducing mesenchyme include aFGF, HGF, EGF, BMP-4, and KGF (Nogawa and Ito, 1995; Post *et al*., 1996; Urase *et al*., 1996), we have demonstrated that aFGF, HGF, EGF, and KGF do not substitute for the mesenchyme (Koike and Shiojiri, 1996; Abe, unpublished data). BMP-4 or other unknown factors may be indispensable in the second hepatic induction. Sonic hedgehog has also been shown to be an endodermal signal inducing BMP-4 and members of the Hox gene family in the gut mesenchyme (Roberts *et al*., 1995,1998; Urase *et al*., 1996; Bellusci *et al*., 1997). The next step will be to determine humoral signaling factors involved in hepatic induction.

Prominent hepatocyte growth occurred when the cell-cell contacts or very local tissue interactions were available between the hepatic endoderm and the lung mesenchyme. The local interactions of the hepatic endoderm and mesenchyme may be mediated by direct cell-cell contacts or via extracellular matrices. In the early phase of liver primordium formation, discontinuous basal laminar laminin expression between the endodermal layer and the hepatic mesenchymal layer occurs (Shiojiri and Katayama, 1987; Cascio and Zaret, 1991), which may permit extensive cell-cell contacts. Thus, the prominent growth of hepatocytes seen in the culture of the present study, in which direct cell contacts were permitted, may be related to the degradation of the basal lamina in normal liver primordium development. In the organogenesis of systems such as lung, kidney and submandibular gland, the importance of cell-matrix interactions has also been indicated (Kadoya *et al*., 1997; Müller *et al*., 1997).

Humoral factors could be effective with direct cell-cell contacts or extracellular matrices in the transfilter experiments with 0.8 and 0.2 μm pore filters, in which direct cell-cell contacts were permitted. Furthermore, the differences in the growth of hepatocytes in the transfilter experiments can be explained by different concentrations of humoral factors passing across the membrane filters. In the transfilter experiments with 0.05 μm pore filters, the concentration of such humoral factors may be too low for the prominent growth of hepatocytes to occur. It is noteworthy that, in the cultures with 0.8 and 0.2 μm pore filters, most hepatocytes proliferated without their direct contact with mesenchymal cells, suggesting that the contact of some endodermal cells with mesenchymal cells is enough to promote their growth. Once some endodermal cells contact mesenchymal cells, they may grow in an autocrine fashion. In any event, the local interactions of the hepatic endodermal cells and lung mesenchymal cells, including the presence or absence of their direct cell-cell contacts, should be examined by transmission electron microscopy in the future.

We observed that large luminal structures were formed by CPSI- and albumin-negative columnar epithelial cells, especially when migration of mesenchymal cells or penetration of cell processes by mesenchymal cells could occur in the transfilter experiments with larger pore sizes. The penetrating mesenchymal cells, and also mesenchymal cells on the other side of the hepatic endoderm across the membrane filter, were preferentially located very close to the typical epithelium. Although it is unknown precisely what kind of organ tissues the epithelial tissue differentiated into (probably the immature stomach judging from the histology), its differentiation may require direct contact with the mesenchymal tissue, which is consistent with tissue interactions during chick stomach development (Takiguchi-Hayashi and Yasugi, 1990). The formation of large luminal structures may be derived from the contamination by the stomach endoderm in the hepatic endoderm (Koike and Shiojiri, 1996) and the use of the lung mesenchyme as an inducer. The lung mesenchyme often constructs typical ducts when directly recombined with hepatic endodermal cells (Koike and Shiojiri 1996).

We also demonstrated that the presence of the hepatic endoderm gave crucial influences on the growth and organization of lung mesenchymal cells. Most lung mesenchymal cells could not survive in vitro in the absence of the hepatic endoderm. The smaller pore sizes of the membrane filters used in the transfilter experiments gave poor growth and low cell density of the mesenchymal cells. These results suggest that humoral factors from the endoderm is indispensable for the survival of the mesenchymal cells in vitro, and that very local interactions including direct cell-cell contacts, or high concentration of the humoral factors pomote their growth and organization.

In conclusion, the second hepatic induction can involve two types of tissue interactions; a humoral one and very local interactions, including direct cell-cell contacts. These data and our culture system may be useful for further studies dealing with mechanisms of the hepatic induction.

ACKNOWLEDGMENTS

The authors are indebted to Dr. W. H. Lamers for his kind donation of anti-CPSI antiserum. We also thank Mr. Kim Barrymore for help with the manuscript. This work was supported in part by a Sasakawa Scientific Research Grant from the Japan Science Society to M. A.

REFERENCES

Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BLM (1997) Involvement of Sonic hedgehog (*Shh*) in mouse embryonic lung growth and morphogenesis. Development 124: 53–63

Cascio S, Zaret KS (1991) Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. Development 113: 217–225

Cereghini S (1996) Liver-enriched transcription factors and hepatocyte differentiation. FASEB J 10: 267–282

Duncan SA, Manova K, Chen WS, Hoodless P, Weinstein DC, Bachvarova RF, Darnell Jr JE (1994) Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. Proc Natl Acad Sci USA 91: 7598–7602

Fukamachi H, Ichinose M, Tsukada S, Kakei N, Suzuki T, Miki K, Kurokawa K, Masui T (1994) Hepatocyte growth factor region specifically stimulates gastro-intestinal epithelial growth in primary culture. Biochem Biophys Res Commun 205: 1445–1451

Fukuda-Taira S (1981) Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm. J Embryol Exp Morphol 63: 111–125

Gaasbeek Janzen JW, Moorman AFM, Lamers WH, Charles R (1985) The development of the heterogeneous distribution of carbamoylphosphate synthetase (ammonia) in rat-liver parenchyma during postnatal development. J Histochem Cytochem 33: 1205–1211

Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS (1996) Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes & Dev 10: 1670–1682

Hentsch B, Lyons I, Li R, Hartley L, Lints TJ, Adams JM, Harvey RP (1996) Hlx homeo box gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. Genes & Dev 10: 70–79

Houssaint E (1980) Differentiation of the mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation. Cell Differ 9: 269–279

Kadoya Y, Salmivirta K, Talts JF, Kadoya K, Mayer U, Timpl R, Ekblom P (1997) Importance of nidogen binding to laminin gamma1 for branching epithelial morphogenesis of the submandibular gland. Development 124: 683–691

Koike T, Shiojiri N (1996) Differentiation of the mouse hepatic primordium cultured in vitro. Differentiation 61: 35–43

Koike T, Yasugi S (1999) In vitro analysis of mesenchymal influences on the differentiation of the stomach epithelial cells of the chicken embryo. Differentiation 65: 13–25

Landry C, Clotman F, Hioki T, Oda H, Picard JJ, Lemaigre FP, Rousseau GG (1997) HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver-enriched transcription factors. Dev Biol 192: 247–257

Le Douarin NM (1975) An experimental analysis of liver development. Med Biol 53: 427–455

McManus JFA (1948) Histological and histochemical uses of periodic acid. Stain Technol 23: 99–108

Müller U, Wang D, Denda S, Meneses JJ, Pedersen RA, Reichardt LF (1997) Integrin $\alpha 8 \beta 1$ is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. Cell 88: 603–613

Nogawa H, Ito T (1995) Branching morphogenesis of embryonic mouse lung epithelium in mesenchyme-free culture. Development 121: 1015–1022

Post M, Souza P, Liu J, Tseu I, Wang J, Kuliszewski M, Tanswell AK (1996) Keratinocyte growth factor and its receptor are involved in regulating early lung branching. Development 122: 3107–3115

Rausa F, Samadani U, Ye H, Lim L, Fletcher CF, Jenkins NA, Copeland NG, Costa RH (1997) The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 β in the developing murine liver and pancreas. Dev Biol 192: 228–246

Roberts DJ, Johnson RL, Burke AC, Nelson CE, Morgan BA, Tabin C (1995) Sonic hedgehog is an endodermal signal inducing *Bmp*-4 and *Hox* genes during induction and regionalization of the chick hindgut. Development 121: 3163–3174

Roberts DJ, Smith DM, Goff DJ, Tabin CJ (1998) Epithelial-mesenchymal signaling during the regionalization of the chick gut. Development 125: 2791–2801

Schuger L, Johnson GR, Gilbride K, Plowman GD, Mandel R (1996) Amphiregulin in lung branching morphogenesis: interaction with heparan sulfate proteoglycan modulates cell proliferation. Development 122: 1759–1767

Shiojiri N, Katayama H (1987) Secondary joining of the bile ducts during the hepatogenesis of the mouse embryo. Anat Embryol 177: 153–163

Takahashi Y, Nogawa H (1991) Branching morphogenesis of mouse salivary epithelium in basement membrane-like substratum separated from mesenchyme by the membrane filter. Development 111: 327–335

Takiguchi-Hayashi K, Yasugi S (1990) Transfilter analysis of the inductive influence of proventricular mesenchyme on stomach epithelial differentiation of chick embryos. Roux's Arch Dev Biol 198: 460–466

Urase K, Mukasa T, Igarashi H, Ishii Y, Yasugi S, Momoi M Y, Momoi T (1996) Spatial expression of *Sonic hedgehog* in the lung epithelium during branching morphogenesis. Biochem Biophys Res Commun 225: 161–166

Vukicevic S, Kopp JB, Luyten FP, Sampath TK (1996) Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). Proc Natl Acad Sci USA 93: 9021–9026

Yang Y, Palmer KC, Relan N, Diglio C, Schuger L (1998) Role of laminin polymerization at the epithelial mesenchymal interface in bronchial myogenesis. Development 125: 2621–2629

(Received December 6, 1999 / Accepted February 3, 2000)